Immunosuppressive and antiproteolytic therapy in vascular diseases

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Immunosuppressive and anti-proteolytic therapy in vascular diseases

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List of abbreviations

(AAA) abdominal aortic aneurysm	(PBS) phosphate-buffered saline
(Ang II) angiotensin II	(pDC) plasmacytoid dendritic cell
(APC) antigen presenting cells	(PDGF) platelet derived growth factor
(apoE-/-) apolipoprotein E deficient	(PMA) phorbol myristate acetate
(BMT) bone marrow transplantation	(RANKL) receptor Activator of NFκB ligand
(BAD) BCL-2-associated agonist of cell death	(ROS) reactive oxygen species
(BMM) Bone marrow-derived macrophages	(SMC) smooth muscle cells
(cat) cathepsin	(TBS) tris buffered saline
(CAV) cardiac allograft vasculopathy	(TF) tissue Factor
(CRP) c-reactive protein	(TGF) transforming growth factor
(EC) endothelial cells	(TIMPs) the tissue inhibitors of metalloproteinases
(ECM) extracellular matrix	(TLR) toll-like receptor
(ELISA) enzyme-linked immunosorbent assay	(TNF) tumor necrosis factor
(EPC) endothelial progenitor cells	(VCAM) vascular cell adhesion molecule
(EvG) Verhoeff-Van Gieson	(WT) wild type
(FACS) fluorescence-activated cell sorting	(α-SMA) α-smooth muscle actin
(FKBP) FK506 binding proteins	
(GM-CSF) granulocyte macrophage colony stimulating factor	
(HE) Hematoxylin and Eosin	
(HED)human equivalent dose	
(HRP) horseradish peroxidase	
(ICAM) intercellular adhesion molecule	
(IFN) interferon	
(IL) interleukin	
(IVUS) intravascular ultrasound	
(LDLr) LDL receptor	
(LFA-1) lymphocyte-function-associated antigen 1	
(LPS) lipopolysaccharide	
(M-CSF) macrophage colony stimulating factor	
(MI) myocardial infarction	
(MFI) mean fluorescence intensity	
(MMF) mycophenolate mofetil	
(MMPs) matrix metallo-proteinases	
(MR) mannose receptor	
(mTOR) mammalian target of rapamycin	
(NFAT) nuclear factor of activated T cells	
(NK) natural killer	
(oxLDL) oxidized low density lipoprotein	
(PAI) plasminogen activator inhibitor	
(PAS) Periodic acid-Schiff	

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Thesis outline and hypothesis

Chapter 1

Inflammation is instrumental in the development and progression of several vascular diseases such as atherosclerosis, neointima formation and abdominal aortic aneurysm (AAA) formation. Immunosuppressive therapy has been considered a promising tool for the treatment of vascular diseases. FK506 is a widely used immunosuppressive drug that inhibits the calcineurin-NFAT signaling pathway. We hypothesize that inhibition of calcineurin/NFAT signaling pathway will protect against inflammation-related vascular pathologies.

Inflammation and extracellular matrix (ECM) degradation are linked at multiple levels during initiation and progression of vascular pathologies and proteolytic arterial remodelling is a key process in AAA, atherosclerosis and neointima formation. One of the most promising therapies to prevent vascular remodelling is to inhibit cathepsin K (catK), a potent lysosomal cysteine protease. Hence it is conceivable that genetic or pharmacological inhibition of catK function will protect against several vasculopathies that are characterized by excessive ECM/elastin degradation.

The introduction is divided into two parts. Part 1 will review the potential of several immunosuppressive drugs to prevent the development and progression of atherosclerosis (chapter 2). Part 2 will review the importance of cathepsin family members in atherosclerosis (chapter 6).

Atherosclerosis is an inflammatory disease. In a previous study, we showed that a low dose of FK506 blocked the progression of murine atherosclerosis. In chapter 3, we will investigate dose-dependent effects of FK506 in a mouse model of collar-induced atherosclerosis as well as on inflammatory parameters relevant to atherosclerosis in an attempt to delineate dose-dependent role of FK506 in inflammatory responses.

Another vascular disease that was reported to show features of inflammation is AAA. AAA is a permanent dilation of the arterial wall in abdominal aorta. Inflammatory processes play a significant role in this vascular pathology. In chapter 4, the effect of low-dose FK506 treatment on AAA formation will be investigated during angiotensin II (Ang II)-induced aneurysm formation.

Intervention in the calcineurin-NFAT pathway in relation to the development of atherosclerosis will be further investigated in chapter 5. As NFATC2 is expressed in several leukocyte subsets and is a crucial transcriptional regulator of numerous inflammatory genes during immune responses, we studied the effect of NFATC2 deficiency in hematopoietic cell lineages in atherosclerosis.

Chapters 7 and 8 will focus on the role of lysosomal cysteine protease-catK in various vascular pathologies. CatK expression was previously found to be elevated in human aortic aneurysm pointing to a role in this vascular disease. In chapter 7, the effect of catK deficiency on Ang II-induced aneurysm formation in the abdominal aorta of apoE^{-/-} mice will be investigated. In chapter 8, the role of catK in two animal models of neointima formation will be investigated.

In chapter 9, the findings in this thesis are discussed and future perspectives are given.

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Drug-induced immuno-modulation to affect development and progression of atherosclerosis – a new opportunity?

Adapated from the following review:

Heeneman S, Donners MM, Bai L, Daemen MJ. Drug-induced immunomodulation to affect the development and progression of atherosclerosis: a new opportunity? Expert review of cardiovascular therapy. Mar 2007;5(2):345-364.

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Abstract

Inflammation and cytokine pathways are crucial in development and progression of atherosclerotic lesions. In this review, the hypothesis that immuno-modulatory drugs are a possible therapeutic modality for cardiovascular disease is evaluated. Therefore, after a short overview of the specific inflammatory pathways involved in atherosclerosis, literature on the effect of several immunosuppressive drugs (immunosuppressive drugs currently used in the prevention of rejection after organ transplant) on the development and progression of atherosclerosis is reviewed.

Immunosuppressive drugs such as mycophenolate mofetil (MMF), sirolimus (rapamycin), cyclosporine A and tacrolimus (FK506), have been studied in atherosclerotic animal models. In general, MMF and sirolimus treatment reduced atherosclerotic lesion size. For cyclosporine A and tacrolimus, results were conflicting, possibly due to the use of different animal models and dosage. A complication of the long-term use of immunosuppressive drugs is the increased risk for opportunistic infections and increased cardiovascular complications (due to development of hypertension and hyperlipidemia). However, a lower dose of immunosuppressive/anti-inflammatory drugs could suffice to decrease the low-grade inflammatory activation in the atherosclerotic lesion without generalized immunosuppressive effects. If such a long-term treatment of a low dose immunosuppressive/anti-inflammatory drug is feasible, a strategy in which the drug is given either as a stabilizing therapy or as a preventive therapy in high-risk patients could be envisioned.

Atherosclerosis is a complex, progressive disease of the large systemic arteries and the leading cause of death in the Western World. This multi-factorial disease is characterized by the accumulation of lipids, cells and extracellular matrix in the vessel wall. Recent research has shown that inflammation and cytokine pathways are crucial in the development and progression of atherosclerotic lesions. Immune cells are already present in early lesions and cytokine pathways are involved in every stage of the disease. Given its nature as an inflammatory disease, immunomodulatory therapies have been proposed for the treatment of atherosclerotic disease. Potential immunosuppressive treatment modalities include specific drugs, chemokine blockades, induction of anti-inflammatory chemokines (e.g. IL-10)¹, gene therapy and immunization/vaccination². In this review, the hypothesis that immuno-modulatory drugs are a possible therapeutic modality for cardiovascular disease is evaluated. After a short overview of inflammatory pathways involved in atherosclerosis, immunosuppressive drugs such as mycophenolate mofetil (MMF), sirolimus (rapamycin), cyclosporine A and tacrolimus (FK506) will be evaluated.

Inflammation in the initiation of atherosclerotic plaques

Atherosclerosis is initiated by dysfunction/damage of the endothelial layer, by factors such as modified LDL, free radicals, hemodynamic stress and hypertension³. The activated endothelial cells (EC) express adhesion molecules, such as E-selectin, P-selectin, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM). In the initial stage, inflammatory mediators such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 α , IL-1 β , IL-18 and interferon (IFN)- γ have been shown to enhance the expression of adhesion molecules⁴. This specifically recruits circulating monocytes

and T-lymphocytes to the lesion site. Pro-inflammatory cytokines, such as IL-1, IL-18, TNF- α and CD40L, induce the expression of monocyte chemotactic protein-1 and IL-8 by EC, smooth muscle cells (SMC) and intimal macrophages^{1, 5-7}. These chemotactic factors mediate the recruitment of circulating monocytes and T-lymphocytes to the lesion site^{8,9}.

In the initial stage of atherogenesis, monocytes differentiate into macrophages under the influence of macrophage colony stimulating factor (M-CSF), a cytokine that is not only produced by macrophages, but also by vascular and stromal cells. Macrophages then become activated by cytokines, which induce the expression of scavenger receptors for the uptake of modified lipoproteins and stimulate macrophage proliferation. IFN- γ , TNF- α and IL-6 regulate the expression of the scavenger receptor-A¹⁰⁻¹², IL-4 upregulates CD36^{13, 14} and transforming growth factor (TGF)- β and TNF- α regulate the expression of the oxLDL receptor (LOX-1)^{15, 16}. Also, the colony-stimulating factors (M-CSF and GM-CSF) can enhance the expression of macrophage scavenger receptors, probably by inducing cytokines. These scavenger receptors mediate the uptake of lipid by macrophages, resulting in foam cell formation. Foam cells can also produce inflammatory mediators such as ILs, TNF- α or IFN- γ , which not only stimulate atherosclerotic plaque progression by the production of growth factors and proteolytic enzymes, but also act in an (auto)regulatory positive feedback loop by producing the cytokines, chemokines and colony stimulating factors described above, that contribute to the chronic nature of the inflammatory response.

The role of inflammatory mediators in plaque progression

Progression of an atherosclerotic plaque is characterized by the formation of a fibrous cap by SMC migration and proliferation, and the development of a necrotic core. An important process in necrotic core formation is cell death, by the process of apoptosis or necrosis. Cytokines also play a role in the regulation of cell survival or apoptosis. IL-1 α , TNF- α and IFN- γ can aggravate the production of reactive oxygen species (ROS) leading either directly to apoptosis¹⁷ or sensitizing EC and SMC for apoptosis by upregulation of Fas-expression¹⁸. Furthermore, TNF- α and IFN- γ can increase macrophage susceptibility to apoptosis induced by peroxisome-proliferator-activated receptor (PPAR)- α and - γ ligands¹⁹. Although certain anti-inflammatory cytokines, such as IL-4, have been shown to promote cell death²⁰, others (such as IL-10) can promote as well as inhibit apoptosis^{21, 22}. This demonstrates that cytokines can exert contradictory effects.

As already mentioned, lesion progression is accompanied by the formation of a fibrous cap, consisting of migrated SMC that proliferate and produce extracellular matrix (ECM) constituents, such as collagen and proteoglycans. Important growth factors for

SMC proliferation include platelet derived growth factor (PDGF), insulin-like growth factor-1 and TGF- β , which can be produced by foam cells. Furthermore, IL-1 and TNF- α produced by foam cells enhance the SMC and EC expression of PDGF, which exerts a variety of functions including stimulation of both proliferation and migration of SMC²³. IFN- γ (which can be induced by IL-12, -15 and/or IL-18) directly inhibits SMC proliferation and collagen synthesis^{24, 25}.

ECM turnover is very important in the progression of atherosclerotic plaques, especially in the determination of plaque stability. An increase in ECM deposition stabilizes the plaque, whereas an increased degradation of the ECM can lead to weakening of the fibrous cap and eventually to plaque rupture. Net matrix deposition thus depends on the balance of ECM synthesis and degradation²⁶. Cytokines not only regulate collagen synthesis, but can also influence the expression of ECM degrading enzymes such as matrix metallo-proteinases (MMPs)^{27, 28}. IL-1 α , TNF- α and CD40L stimulate MMP-1, -2, -3, -7, -8, -9, -11 and -13 expression in EC, SMC and macrophages/foam cells^{29, 30}. Interestingly, IFN- γ attenuates both collagen synthesis and degradation, indicating again the multi-functional effects of cytokines.

Inflammation and plaque rupture

Inhibition of SMC proliferation, SMC apoptosis, inhibition of matrix synthesis, and increased matrix degradation may affect integrity and strength of the fibrous cap and can lead to rupture of an atherosclerotic lesion and exposure of the necrotic core to the blood. The necrotic core is highly thrombogenic due to its lipid content and the presence of pro-coagulant factors, such as Tissue Factor (TF)³¹. These factors together with (anti-)fibrinolytic factors, such as plasminogen activators, urokinase-type plasminogen activator, tissue plasminogen activator and plasminogen activator inhibitor (PAI) influence plaque rupture. Cytokines can also regulate the expression of pro-coagulant and anti-coagulant factors. IL-1, TNF- α and CD40L can induce TF expression in macrophages, EC and SMC^{32, 33}. Furthermore, IL-1 α and TNF- α can enhance the expression of PAI-1³⁴, whereas IFN- γ has the opposite effect³⁵. Thus, by governing the expression of pro-coagulant, pro- or anti-fibrinolytic factors, cytokines may regulate the thrombogenicity of atherosclerotic plaques.

Balance of pro-inflammatory and anti-inflammatory mediators

Anti-inflammatory cytokines, such as IL-4 and -10 can reduce the expression of adhesion molecules (ICAM & VCAM)³⁶, chemotactic factors (IL-8)³⁷, growth factors (through inhibition of IL-12, -15 and -18), proteolytic enzymes (MMPs)^{38, 39} and pro-coagulation factors (TF)³³. Therefore, these cytokines can counteract the effects of pro-inflammatory cytokines and possibly protect against atherosclerosis formation.

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Furthermore, mediators other than cytokines can influence the inflammatory response. Angiotensin II (AngII), for example, has been shown to exert several pro-inflammatory and pro-atherogenic effects, such as increasing free radicals (ROS), stimulation of SMC proliferation and expression of adhesion molecules, scavenger receptors, cytokines and chemotactic factors ⁴⁰.

The balance between pro-inflammatory and anti-inflammatory cytokines, chemokines and growth factors determines the net effect of these mediators. A key regulatory event is the differentiation of CD4⁺ T-cells into either a Th1 or Th2 subtype. The Th1 response typically involves pro-inflammatory and mainly pro-atherogenic mediators, such as IFN- γ , TNF- α and IL-1, whereas the Th2 response favors the production of anti-inflammatory and anti-atherogenic cytokines IL-4 and -10. The differentiation of CD4⁺ cells into Th1 or Th2 phenotypes is also regulated by cytokines ⁴¹. IL-12 induces a Th1 response, IL-10 a Th2 response. These Th1 and Th2 cytokines are cross-regulatory, since IL-10 inhibits the Th-1 response, whereas IFN- γ inhibits Th2.

It has been shown that in the atherosclerotic plaque, inflammatory mediators of the Th1 response dominate ⁴². Pro-inflammatory T-cell cytokines (IL-2 and IFN- γ) were shown to be highly expressed, whereas the anti-inflammatory cytokines IL-4 and -5 were rarely observed in the plaques.

In conclusion, atherosclerosis is a very complex, chronic inflammatory disease, initiated by a response to injury and mediated by a constant interplay between (inflammatory) cells and inflammatory mediators. Cytokines play an important regulatory role in the recruitment of inflammatory cells (by modulating the expression of adhesion molecules and chemotactic factors), foam cell formation (by inducing expression of scavenging receptors), fibrous cap formation (by regulating SMC migration, proliferation and matrix turnover), necrotic core formation (by regulating cell survival, death or phagocytosis) and thrombogenicity of the plaques (by modulating the expression of pro-coagulant, pro- or anti-fibrinolytic factors).

Established immunosuppressive drugs– immuno-modulation as a ‘side-effect’.

Established immunosuppressive drugs can, at least to a certain extent, have anti-inflammatory effects. It has been suggested that these pleiotropic immunomodulatory effects contribute to the clinical benefits observed. Here we will focus on the immuno-modulating effects of MMF, sirolimus, cyclosporine A and tacrolimus.

Mycophenolate mofetil (MMF)

MMF is a potent immunosuppressant that is currently in use in the prevention of acute rejection after organ transplantation. MMF is converted in the liver to the active

compound mycophenolic acid, which is a reversible, potent, non-competitive inhibitor of monophosphate dehydrogenase, the central, rate-limiting enzyme in de novo purine synthesis. As lymphocytes only have the capacity for de novo synthesis of guanosine nucleotides, MMF has a potent anti-proliferative effect on these cells by depletion of the intracellular guanosine nucleotide pool ^{43, 44}. In addition, MMF can inhibit expression of adhesion molecules on lymphocytes and EC and increase T-cell apoptosis ⁴⁴. MMF was first used in clinical trials for renal transplantation, and since 1993 MMF has also been used in cardiac transplantation. In a recent review in which clinical trials in 28 centers were analyzed, MMF reduced mortality and the incidence and severity of cardiac transplant rejection. In addition, MMF was able to better preserve coronary artery luminal area 1 year after transplantation, suggesting that MMF could reduce progression of cardiac allograft vasculopathy, a well-known complication and cause of organ loss ⁴⁵. In line with this, cardiac transplant patients, treated with MMF had lower plasma levels of CRP compared to patients receiving other immunosuppressive drugs ⁴⁶.

The effect of MMF and its immuno-modulating properties in atherosclerosis have been investigated in a limited number of studies. Although all studies used a rabbit model (in which a high fat diet induces fatty streak development in the aorta), the effect of MMF was clearly beneficial with a reduction in lesion development and with lesions containing less SMCs and macrophages ⁴⁷⁻⁴⁹. Given its success in transplant patients and favorable side-effect profile, MMF could be a good candidate for an immuno-modulating drug used to prevent progression of atherosclerosis, although more animal and human studies are needed to evaluate the cardiovascular effects of MMF.

Sirolimus (Rapamycin)

Sirolimus, also known as rapamycin, is a macrolide and the metabolic substrate of the fungus *Streptomyces hygroscopicus*. Developed initially as an anti-fungal antibiotic, it was later discovered that it also possessed potent immunosuppressive activities and was further developed for use in renal transplant rejection. Sirolimus has a structure similar to the bacterial macrolide FK506 (tacrolimus), and binds to the same family of immunophilins, the FK506 binding proteins (FKBP-12 specifically). While the FK506-FKBP-12 complex will inhibit calcineurin (see next paragraph), the sirolimus -FKBP-12 complex will inhibit the kinase mTOR (mammalian target of rapamycin). mTOR is a 289 kD protein, evolutionarily related to lipid kinases, and has protein serine/threonine kinase activities ⁵⁰. mTOR regulates proteins that are critical in cell cycle regulation (p70^{s6k}, cyclin-dependent kinases, their cyclin partners, the cyclin dependent inhibitory proteins p21 and p27). The inhibition of mTOR by sirolimus will inhibit cell cycle progression in a number of cell types, including T and B-lymphocytes. The effectiveness

of sirolimus as an immunosuppressive drug is due to the fact that IL-2 dependent T-cell proliferation is dependent on mTOR signals to drive T-cell proliferation⁵¹. Activation of mTOR also regulates proliferation of SMCs and EC in response to growth factors. The latter explains the successful use of sirolimus in coated stents in the prevention of in-stent restenosis in various clinical trials⁵². As mentioned above, sirolimus has been initially developed for use in renal transplant rejection. In several clinical trials, sirolimus has been successful in preventing acute rejection and improving long term renal function (1 year)⁵¹. In a prospective study, patients with coronary allograft vasculopathy were treated with sirolimus (on a background of 'standard' immunosuppression). In the follow-up of 2 years, the patients treated with sirolimus developed significantly less adverse events (death, myocardio infarction (MI), need for bypass surgery)⁵³.

Given these anti-proliferative and immunosuppressive properties, the use of sirolimus in the treatment of atherosclerosis has been studied in experimental models. In all mouse studies⁵⁴⁻⁶⁰, sirolimus reduced atherosclerotic lesion size. If investigated, this was paralleled by a reduction in inflammatory parameters, either systemic or intralesional. In 2 studies, sirolimus affected serum lipid levels, increasing triglyceride levels in one study⁵⁵, and increasing both LDL and HDL levels in the other⁵⁸. These changes in lipid levels are disadvantageous if sirolimus is to be used as a long term treatment for atherosclerosis, and indeed clinical trials thus far showed that hyperlipidemia is evident in most patients treated with sirolimus⁵¹. In addition to its anti-proliferative and anti-inflammatory properties, sirolimus was able to inhibit lipid uptake and increase cholesterol efflux from VSMCs, which could contribute to the anti-atherosclerotic effects of sirolimus⁶¹. However, there are no data available from clinical trials showing the effect of sirolimus on native atherosclerosis. Of the same family as sirolimus, everolimus is an orally active immunosuppressive and anti-proliferative compound. Everolimus, although did not alter total plasma cholesterol levels in LDL receptor (LDLr) -/- mice fed with western diet, was found to significantly reduce atherosclerosis lesions⁶². In addition, everolimus markedly decreased lesion macrophage content and this effect was associated with a reduction in plaque area and occurred in the absence of changes in plasma cholesterol concentrations⁶³. Pretreatment with everolimus lowered monocyte chemotaxis in response to various chemotactic factors. This may explain the reduced macrophage content in atherosclerotic lesions. Similarly, another study reported that implantation of everolimus eluting stents led to a reduction in macrophage content without altering the amount of smooth muscle cells in atherosclerotic lesions⁶⁴. Subsequent in vitro studies indicated that everolimus only induced cell death in macrophages. This study indicated that stent-based delivery of everolimus may be a promising novel strategy for treatment of vulnerable atherosclerotic plaques⁶⁴. However further studies are needed to validate this hypothesis.

Cyclosporine A and tacrolimus (FK506)

Although structurally unrelated, cyclosporine A and tacrolimus both inhibit calcineurin (protein phosphatase 2B), a serine/threonine protein phosphatase directly regulated by Ca^{2+} and calmodulin. Cyclosporine A is a lipophilic undecapeptide, whereas tacrolimus is a macrolide antibiotic isolated from *streptomyces tsukubaensis*. They bind to different immunophilins to exert their effects: cyclosporine A complexes with cyclophilin and tacrolimus binds to FKBP (FKBP12 is the major FKBP for tacrolimus/FK506). Both drug-immunophilin complexes inhibit the phosphatase activity of calcineurin and thereby prevent the dephosphorylation and nuclear transport of the NFAT (nuclear factor of activated T-cells) family of transcription factors. This results in a suppression of NFAT regulated genes, such as IL-2, IFN- γ and TNF- α , and in turn the elimination of inflammatory reactions^{65, 66}.

Given these anti-inflammatory properties, cyclosporine A or tacrolimus are currently the primarily used immunosuppressive therapies after organ transplantation. The introduction of cyclosporine A in 1982 markedly improved clinical outcome for patients with a heart transplantation, as 3 year survival increased by ~40-70%. The efficacy of cyclosporine A and tacrolimus have been compared in several large clinical trials. Although survival and incidence of acute rejection are generally the same, tacrolimus is often preferred by clinicians due to lower incidence of hyperlipidemia and hypertension⁵¹. Despite improvements in preservation of the transplanted organ due to cyclosporine A and tacrolimus, the incidence of coronary artery disease in heart-transplant patients (1-18% in year 1, 20-50% after 3 years) has not decreased in the past 25 years. Allograft vasculopathy is an accelerated form of organ disease and a long term complication of patients with a heart or kidney transplantation⁶⁷. Allograft vasculopathy affects both intramural and epicardial coronary arteries and veins. The etiology is not similar to atherosclerosis (as it affects veins as well), although a non-denuding arterial injury is thought to be a major factor in its development. Immunosuppressive drugs have been proposed as causative factors to allograft vasculopathy, either directly due to toxicity or indirectly due to hyperlipidemia and hypertension⁶⁸.

Despite the apparent discrepancy in the immunosuppressive/anti-inflammatory effect versus deterioration of allograft vasculopathy by cyclosporine and tacrolimus treatment, the effect of these drugs on atherosclerosis and arteriosclerosis have nevertheless been investigated in animal models of transplant arteriosclerosis, denudation-inflicted vascular injury and atherosclerosis. The data of these studies⁶⁹⁻⁷⁶, however, are rather contradictory, possibly due to specific animal models used and large differences in dosages. The studies in models of transplant arteriosclerosis in general show a protective effect^{72, 73, 75}. In atherosclerosis, however, tacrolimus or cyclosporine either

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increased^{69, 71, 74} or decreased lesion size^{70, 76}. None of these studies used the same model or dose. The dosage, however, could be of critical importance as many of the side-effects of tacrolimus and cyclosporine are reported in patients on high dose regimes for maximal suppression of the inflammatory response against the 'foreign' transplanted organ. In atherosclerosis, however, the inflammatory reaction is more local and sustained over longer periods of time, especially in the initial phases. In a study from our laboratory⁷⁶, using a low dose of tacrolimus/FK506 (0.05 mg/kg/d) in two models of atherosclerosis (collar-induced atherosclerosis and native atherosclerosis in apoE^{-/-} mice), atherosclerotic plaque development was decreased in both models in the tacrolimus-treated mice, without affecting plasma lipid levels and without systemic side-effects. Moreover, tacrolimus treatment increased collagen content and decreased local inflammatory parameters, suggesting that tacrolimus induced a more stable plaque phenotype. The chronic (subcutaneous) administration of 0.05 mg/kg/d resulted in (whole) blood tacrolimus concentrations of ~0.2 ng/ml, which is considerably lower compared to levels achieved in transplant patients (15-20 ng/ml)⁷⁷. In addition, there is strong evidence that the local expression of different isoforms of immunophilins (such as FKBP12) may determine the effect of tacrolimus on a particular tissue⁷⁸. This implies that the same dose of tacrolimus could affect different cell types differently⁷⁶. Interestingly, FKBP12 was found to be upregulated in human coronary atherectomy tissue retrieved by helix cutter⁷⁹, suggesting that the atherosclerotic human vascular wall may be more sensitive to tacrolimus⁷⁹. Combined, data from literature and our own data suggest that tacrolimus exerts its regulatory actions on plaque phenotype primarily at the level of the vessel wall itself and not via a systemic immuno-modulatory effect.

Perspectives for long term (oral) use of immuno-modulatory drugs

As suggested by several experimental studies, in animal models as well as in the limited number of patient studies, immuno-modulatory drugs could become a potential therapy for atherosclerosis. Table 2.1 gives an overview on how these drugs could interact with the different stages of atherosclerosis (as related to the events described in the first part of this review). Clearly, all drugs can interact in multiple events, both in the initiation as well as the progression/rupture phase. However, there are several issues to consider, both in favor of, as well as against the use of immuno-modulatory therapy in patients with cardiovascular disease.

The use of systemic immunosuppressive therapy fits within the current paradigm that atherosclerosis is a systemic disease and that, although thrombotic occlusion of a single ruptured plaque can be the cause of a cardiovascular event, few vulnerable lesions are often present in the same patient. This has been shown in several studies using different approaches (intravascular ultrasound (IVUS), autopsy, angiography,

angiography and thermography studies (reviewed in ⁸⁰). Mariello et al ⁸¹ also showed that, in patients who died of an acute MI, more vulnerable lesions were present (~7/patient, compared with ~1 in patients with stable angina and control patients). Acute MI patients showed a diffuse and active inflammatory process in the entire coronary tree, also in stable lesions distant from the culprit lesion. Buffon et al ⁸² also showed the presence of widespread coronary inflammation in patients with unstable angina. A recent study by Rioufol et al ⁸³ used IVUS to follow ruptured non-culprit lesions on a background therapy of statins and anti-platelet drugs. It was shown that 50% of the lesions healed after a follow-up of 22 months. Although only small non-culprit lesions were studied (as these were not treated by interventional cardiology), this study did show that lesions can heal with medical therapy. Thus, systemic drugs that can stabilize or regress the multiple vulnerable lesions are of great benefit. Moreover, given the possibility of widespread coronary inflammation in vulnerable patients, the impact of anti-inflammatory/immunosuppressive drugs could be substantial.

An important draw-back of the long-term use of immuno-modulatory drugs is the suppression of 'normal' immunoregulatory functions and increased incidence of either bacterial or viral infections. In transplanted patients, complications or death due to cytomegalovirus, Epstein-barr virus or hepatitis C are a major source of mortality or morbidity ⁸⁴.

Although we postulated in the previous paragraph that anti-inflammatory/immunosuppressive drug could be a promising therapy for multiple vulnerable lesions, the draw-back of the long-term use of immuno-modulatory drugs is an increased incidence of cardiovascular complications after heart transplantation. This is reflected by the fact that accelerated coronary disease is the leading cause of death in heart transplanted patients >1 year post transplant. In addition, death due to cardiovascular disease, while the transplanted organ is still functioning, is a major cause of death following renal and cardiac transplantation. As shown in table 2.2, most of the drugs described in this review increase one or more risk factors such as hyperlipidemia, dyslipidemia, hypertension and diabetes mellitus ⁶⁸. MMF displays the most favorable side-effect profile.

Clearly there is discrepancy between the possible protective effect of immunosuppressive therapy against vulnerable lesions and the cardiovascular disease after long term immunosuppressive therapy. A point to consider that might explain this discrepancy is the dosage of the drug that might have pivotal importance in both the suppression of normal regulatory immune function and cardiovascular side-effects. It is expected that a lower dose of immunosuppressive drugs as currently used for the suppression of solid organ rejection, is needed for the suppression of the more low-grade continuous/episodic inflammatory activation as seen in the coronary vasculature of patients with

unstable angina. It is encouraging that in an animal study from our own laboratory, a low dose of systemic tacrolimus was able to inhibit progression of atherosclerosis in two models, with no apparent effects on systemic immunological parameters⁷⁶. If a long-term treatment of a low dose immunosuppressive drug is feasible, a strategy in which the drug is given either as a stabilizing therapy or as a preventive therapy in high-risk patients could be envisioned.

Summary

Recent research has shown that inflammation and cytokine pathways are crucial in the development and progression of atherosclerotic lesions. Immune cells are already present in early lesions and cytokine pathways are involved in every stage of the disease. Given its nature as an inflammatory disease, immunomodulatory therapies have been proposed. Several immunosuppressive drugs are already used in the clinic, mostly to suppress organ rejection in patients with organ transplants. The question is whether these drugs could also be used to suppress inflammatory activation that is associated with initiation and progression of atherosclerosis and become a potential therapeutic modality. Immunosuppressive drugs such as MMF, sirolimus, cyclosporine A and tacrolimus and their concomitant anti-inflammatory effects, have also been studied in atherosclerotic animal models. MMF has been studied only in rabbit models, in which an atheroprotective potential has been shown. With respect to sirolimus, more studies are available, and a consistent reduction in lesion size was present after treatment. For cyclosporine A and tacrolimus, results in animal studies are less consistent, with both an increase as well as a decrease in atherosclerotic burden. A complication of the long-term use of these immunosuppressive drugs (MMF, sirolimus, cyclosporine A, tacrolimus) in patients with transplanted organs, is the increased risk for opportunistic infections and increased cardiovascular complications (due to development of hypertension, and hyperlipidemia). However, a lower dose of immuno-modulating drugs could suffice to decrease the low-grade inflammatory activation in atherosclerotic lesions without generalized anti-inflammatory or immunosuppressive effects. For tacrolimus, it was shown that a low-dose regime did inhibit progression of atherosclerosis in two mouse models of atherosclerosis, with no apparent effects on systemic immunological parameters⁷⁶. If a long-term treatment of a low dose immuno-modulating drug is feasible, a strategy in which the drug is given either as a stabilizing therapy or as a preventive therapy in high-risk patients could be envisioned.

Table 2.1 : Overview of the effect of the different drugs on plaque stage/ events

		MMF	Sirolimus	CsA	Tacro.
Plaque initiation	EC activation/ Expression adhesion molecules	↓	↓	↓	↓
	Recruitment inflammatory cells	↓	↓	↓	↓
	Monocyte to macrophage transition	↓	?	?	?
	Activation macrophages (lipid uptake)	?	?	?	↓
Plaque progression/ Plaque rupture	SMC migration/ proliferation	↓(prol)	↓(prol)	↑↓(prol)?	=/?
	ECM remodelling	↓ ? indirect	↓ ? indirect	?	↑
	Apoptosis	T-cell ↑	?	↓	↓(smc)
	Angiogenesis	?	↓	↓	?
	Thrombosis	?	?	?	?

prol. = proliferation, SMC = smooth muscle cell, tacro. = tacrolimus, CsA=cyclosporine A

Table 2.2: Side-effects of immuno-modulatory drugs

	Changes in lipid metabolism	Hypertension	Diabetes	Gastro- intestinal problems	Renal dysfunction
MMF	NR	NR	NR	↑ ↑	NR
Sirolimus	↑ ↑ ↑	NR	NR	↑ ↑	NR
Cyclosporine A	↑ ↑	↑ ↑ ↑	↑	↑	↑ ↑
Tacrolimus	↑ ↑	↑ ↑	↑ ↑	↑ ↑	↑ ↑

Drugs as reported for use in patients (used for prevention of organ rejection (high dose regimes)).

NR = Not reported

Gastro-intestinal problems: Nausea, diarrhea and vomiting for the other drugs.

Chapter 2

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3

Differential effect of FK506 dosage on the development of atherosclerosis due to a dose-dependent shift in Th1/Th2 balance

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Abstract

Aims: Previous studies showed both pro- and anti-atherogenic effects of immunosuppressant drug FK506 on atherosclerosis. As these divergent/paradoxical results of FK506 may at least in part be attributable to differences in FK506 dosing, we have in the current study, assessed dose dependent effects of FK506 on atherosclerotic lesion formation as well as on inflammatory parameters relevant to atherosclerosis.

Methods and results: Unlike low dose FK506, high dose FK506 did not protect against atherosclerosis in apoE^{-/-} mice. While both low and high dose FK506 treatment significantly reduced systemic CD3⁺ and CD4⁺CD25⁺ T-cell populations in lymph node, only high dose treatment led to a suppression of the FoxP3 regulatory T cell population in spleen. Conditioned media from splenic CD4⁺ T cells of low dose FK506-treated mice showed sharply increased IL-4/IFN- γ and IL-10/IFN- γ ratios suggestive of Th2 skewing, whereas high dose FK506 treatment did not induce such a response. In vitro studies with FK506 exposed CD4⁺ T cells, Jurkat T cells and PMA-differentiated THP1 cells confirmed the in vivo findings.

Conclusions: These results identify dosage as a critical factor in the anti-atherogenic activity of FK506, probably by differentially skewing the Th1/Th2 balance. The fact that low dose FK506 treatment induces a minor but decisive Th2 shift suggests that it can be particularly effective for long-term therapy in Th1 dominated immune disorders such as atherosclerosis.

Introduction

A potent immunosuppressive drug, FK506 (tacrolimus) is widely used in patients following transplantation. At a molecular level, FK506 binds to the cytosolic FK506 binding protein 12 (FKBP12)-member of FKBP family¹. In T lymphocytes, FK506 interacts with FKBP-12 to form a FK506-FKBP-12 complex². This complex blocks calcineurin, a Ca^{2+} -calmodulin-regulated protein phosphatase. As a consequence, calcineurin fails to dephosphorylate amongst others cytosolic nuclear factor of activated T cells (NFAT), thereby inhibiting its translocation to the nucleus. NFAT is one of the most important transcriptional factors involved in the activation of cytokine genes such as IL-2. In addition, NFAT partners with the transcription factor AP-1 (Fos-Jun) in the nucleus of “effector” T cells. As a consequence, FK506 will inhibit both NFAT and NFAT-AP1 function in transactivating cytokine genes, genes that regulate T cell proliferation and other genes that orchestrate an active immune response³.

Atherosclerosis is a chronic inflammatory disease in which NFAT responsive cytokines such as IL-2 are deemed to play a prominent role. In a previous study, we showed that FK506 at a low dose of 0.05 mg/kg/day beneficially affected the progression of murine atherosclerosis, reducing plaque area and increasing cell density and collagen content⁴. Intriguingly and in contrast to our findings, Matsumoto et al reported that FK506 treatment deteriorated atherosclerosis in cholesterol-fed rabbits⁵. In their study, a relatively high dose of FK506 was applied. Conceivably dose-specific effects of FK506 on cell subsets relevant to atherosclerosis⁶, and most notably leukocytes, may explain these paradoxical findings. For clinical practice, it is important to comprehend the mechanisms underlying these potentially dose dependent effects of FK506 on atherosclerosis, in particular as in organ transplant recipients a high dose regime was seen to be associated with substantial side-effects^{7, 8}. Therefore, in this study, we investigated effects of low and high dose FK506 treatment on atherosclerosis and atherosclerosis related immunomodulation in apoE^{-/-} mice.

As our data pointed to dose-dependent immunoregulatory effects of FK506, *in vivo* and *in vitro* experiments were designed to address this in more detail. Firstly, we studied whether *in vivo* low/high dose FK506 treatment differentially affected T cell differentiation and activity in spleen, lymph node and peripheral blood. Secondly, splenic gene expression of I κ B and NF κ B responsive gene such as ICAM-1 and P-selectin were analyzed by real-time PCR. Finally, the effect of *ex vivo* and *in vitro* treatment of low/high dose FK506 on cytokine secretion by T cells and PMA-stimulated THP1 cells were investigated.

Materials and Methods

In Vivo studies

Mouse model of collar-induced atherosclerosis

Animal experiments were approved by the regulatory authority of the University of Maastricht and were performed in compliance with the Dutch government guidelines. Male apoE^{-/-} mice (14 weeks old, C57Bl6 background) were fed a Western-type diet throughout the experiment. Carotid atherosclerotic lesions were induced by silastic tubing (collar) around carotid artery, as described by von der Thüsen et al⁹. To assess the effect of FK506 on lesion initiation, one week after collar-placement, osmotic minipumps (Alzet type 2004) containing FK506 (Fujisawa GmbH) or PBS were placed subcutaneously to achieve pumping rate of either 0.05 mg/kg/day or 1 mg/kg/day. Mice were sacrificed 4 weeks after pump placement.

Tissue harvesting and analysis

Cross-sections from the right common carotid artery after *in situ* perfusion-fixation were processed for morphometric analysis as described previously¹⁰. Kidneys cross-sections (3 µm thick) were stained with Periodic acid-Schiff (PAS) for kidney toxicity analysis. (Immuno)histochemical stainings were performed to detect macrophages (α-Mac3 Ab) and collagen (picrosirius red) as described previously¹⁰. FoxP3 immunohistochemistry staining was performed by incubating sections with rat anti-mouse polyclonal Ab against the FoxP3 protein (1:50, eBioscience).

Fluorescence-activated cell sorting (FACS)-analysis

ApoE^{-/-} mice were treated with FK506 or phosphate-buffered saline (PBS) using osmotic minipumps as described above. After 4 weeks, mice were sacrificed. Spleen, lymph node cells were isolated and used to make single-cell suspensions. Erythrocytes in peripheral blood and spleen were removed by hypotonic lysis with NH₄Cl. Cells were incubated first with anti-CD16/32 (eBioscience) to block Fc binding and subsequently incubated with anti-CD3-FITC, anti-CD8-Pacific blue, anti-CD25-APC (eBioscience) and anti-CD4-PerCp (BD-Biosciences Pharmingen). Foxp3 positive cells were detected with PE anti-mouse/rat Foxp3 Staining Set, according to the manufacture's instruction (eBioscience).

Quantitative RT-PCR

RNA was extracted from the spleen lysate with Nucleospin RNA II kit (MACHEREY-NAGEL). cDNA was generated with iScript™ CDNA synthesis kit (BIO-RAD). Real-time PCR was done with a Taqman IQ™ SYBR Green Super Mix (BIO-RAD). Primer sequences for FoxP3, Iκβ-α, P-selectin, ICAM-1 and cyclophilin were shown in table 3.1.

Table 3.1. Primer sequence

	<i>Forward primer (5'-3')</i>	<i>Reverse primer (5'-3')</i>
FoxP3	CCAGTCTGGAATGGGTGTCC	CTGCTTGGCAGTGCTTGAGA
I κ B- α	TGGAAGTCATTGGTCAGGTGAA	CAGAAGTGCCTCAGCAATTCCT
P-selectin	GGTATCCGAAAGATCAACAATAA GTG	GTTACTCTTGATGTAGATCTCCAC ACA
ICAM-1	GGACCACGGAGCCAATTTC	CTCGGAGACATTAGAGAACAATGC
cyclophilin	CAAATGCTGGACCAAACACAA	TTCACCTTCCCAAAGACCACAT

Ex vivo and in vitro study on low dose FK506 mechanism (table 3.2)

Table 3.2. Ex vivo and in vitro study on low dose FK506 mechanism

<i>Experiment</i>	<i>Treatment</i>	<i>FK506 dose</i>	<i>Isolation</i>	<i>PMA & Ionomycin in vitro</i>
1	In vivo	Low dose: 0.05 mg/kg/day High dose: 1mg/kg/day	CD4 ⁺ T cells	1ng/ml & 500nM 4ng/ml & 500nM
2	In vitro	Low dose: 20ng/ml High dose: 2000ng/ml	CD4 ⁺ T cells	16ng/ml & 2000nM
3	In vitro	Low dose: 20ng/ml High dose: 2000ng/ml	Jurkat T cells	8ng/ml & 1000nM
4	In vitro	Low dose: 20ng/ml High dose: 2000ng/ml	PMA differentiated THP1 cells	PMA: 120ng/ml LPS: 25ng/ml
5	In vitro	Low dose: 20ng/ml High dose: 2000ng/ml	Nucleus of Jurkat T cells	8ng/ml & 1000nM

FK506 treatment on CD4⁺ T cells

CD4⁺ T cells were isolated from splenocytes of FK506 treated or untreated mice using the Negative Isolation Kit (Dynal, Biotech ASA). The purity of CD4⁺ T cells was $87 \pm 5\%$ as evaluated by FACS (data not shown).

CD4⁺ T cells were incubated with PMA (Sigma) and Ionomycin (Sigma) for 48 hours. IL-4, IL-10 and IFN- γ in supernatants were assayed by enzyme-linked immunosorbent assay (ELISA) using antibodies according to the manufacturer's instructions (BD Bioscience). IL-4:IFN- γ and IL-10: IFN- γ ratio were calculated and used as an index of Th2/Th1 skewing.

Effects of FK506 on Jurkat T cells

Cytokine analysis was performed on human T lymphocytic Jurkat cells to further clarify the mechanism. Jurkat T cells were pretreated with FK506 at a dose of 0, 20, and 2000 ng/ml for 1 hour. Cells were then stimulated with PMA and ionomycin for 48 hr. IFN- γ and IL-10 in supernatant were assayed by ELISA using antibodies according to the manufacturer's instructions (Sanquin).

Effect of FK506 on PMA-differentiated THP1 cells

The human promonocytic cell line THP-1 was differentiated in the presence of PMA for 72 h. Differentiated THP1 cells were then treated for 24h with FK506 at a concentration of 0, 20 and 2000 ng/ml for 24 hours. Cells were then treated for 8 hours with LPS (25ng/ml) in the presence of the original concentration of FK506. Supernatants were harvested and analyzed for human IL-10 and IFN- γ content by ELISA according to the manufacturer's instructions (Sanquin).

Effects of FK506 on NFATC1 and NFATC2 expression on nucleus of Jurkat T cells

Preparation of nuclear extracts and Western blot analysis

Nuclear and cytoplasmic extracts from Jurkat T cells were prepared using NE-PER nuclear and cytoplasmic reagents (Pierce) according to the manufacturer's protocol. Protein concentration of nuclear extracts was determined by a Bio-Rad protein assay (Bio-Rad). Nuclear extracts (30 μ g) of each sample were loaded onto 7.5% SDS polyacrylamide gel under reducing conditions, and transferred to nitrocellulose transfer membrane (Whatman). Membranes were blocked with 2% Marvel/TBS-Tween and incubated with mouse anti-TATA binding protein TBP antibody (1/1000, abcam) in 2% Marvel/ tris buffered saline (TBS)-Tween (nuclear loading control), mouse anti-NFATC1 and mouse anti-NFATC2 (1/2000, Santa Cruz). Membranes were washed with TBS-Tween, and incubated with rabbit anti-mouse polyclonal Ab labelled with (horseradish peroxidase) HRP (1/2000, Dako, Denmark A/S) in 2% Marvel/TBS-Tween. Protein bands were visualized by chemiluminescence, and the signal intensity quantified by the Quantity One imaging system (Bio-Rad).

Viability analysis after FK506 treatment

Isolated splenocytes from apoE^{-/-} mice were cultured under the same conditions as CD4⁺ T cells. Cells were exposed to FK506 treatment ranging from 0.2ng/ml to 20 μ g/ml for 72 hours. Viable cells were counted using trypan blue.

Figure 3.1 Influence of low/high FK506 treatment on mice weight.

Statistical analysis

All results were expressed as mean $\pm$ SEM. Differences between values were considered significant at $p < 0.05$ using Mann-Whitney U test.

Results

Low-dose FK506 inhibits collar-induced atherosclerosis whereas high-dose did not

Mice weight was not affected by low/high dose FK506 treatment as shown in figure 3.1. Concentrations of FK506 in blood were essentially similar as described in our previous study⁴. To determine the effect of FK506 on the histological changes in kidneys, histological analysis was performed using PAS staining. Focal microaneurysm was present in glomeruli at similar frequency in the low/high dose group. There was no histological evidence of tubular toxicity. Acute (indicated by isometric vacuolization and thrombotic mesangiopathy) or chronic kidney toxicity (indicated by arteriolar hyalinosis and tubularinterstitial fibrosis atrophy) were not observed.

In keeping with our earlier study⁴, plaque size in low dose FK506-treated mice was significantly lower (72.1%, $p=0.05$) compared to untreated controls. Remarkably, plaque sizes of control and high dose-treated mice were essentially similar (Figure 3.2A). There was no significant difference in macrophage content between control, low and high dose group (figure 3.2B). Lipid core size in the low dose group tended to be decreased compared with control group ($p=0.075$), an effect that was not observed after high dose treatment (figure 3.2C). In addition, both low and high dose treatment did not affect collagen content (figure 3.2D). Figure 3.2E show representative HE stained cross-sections of control, low dose versus high dose FK506-treated mice, respectively. Full color figures of HE stained cross-sections are shown on page 172.

Low/high FK506 treatment suppressed peripheral immune activity

To examine whether FK506 influenced lesion development indirectly by altering peripheral immune activity, single-cell suspensions of spleen, lymph nodes and blood were analyzed to determine the amount of T cells by FACS (figure 3.3). The CD3⁺

Figure 3.2 Morphological and morphometric analysis of the effect of low and high dose FK506 treatment on collar-induced atherosclerotic plaque development in common carotid arteries of apoE^{-/-} mice. (A) the average of intima volume *p = 0.05, control versus low; (B) amount of macrophages in plaques, macrophage content was expressed as Mac3-positive cell numbers relative to total number of plaque cells of the section with the largest plaque area. (C) lipid core area relative to plaque area (p = 0.075, control versus low; p = 0.083, low versus high). (D) collagen content in plaques, collagen content stained by picosirius red and are expressed relative to the plaque area. E shows representative HE stained cross-sections of right carotid artery of control (n=10), low dose (n=9) versus high dose FK506 (n=10)-treated mice, respectively. Full color figures of HE stained cross-sections are shown on page 172

T cell content in spleen, lymph node and blood was significantly reduced after low and high dose FK506 treatment (figure 3.3A). The relative abundance of CD4⁺ cells within the CD3⁺ population in lymph node was significantly reduced after high dose FK506 treatment, whereas low dose did not alter the CD4⁺ cell content (figure 3.3B). Furthermore, the CD4⁺ to CD8⁺ ratio in lymph node was significantly reduced with both low and high dose of FK506 treatment (figure 3.3D). This was reflected by a reduction in CD4⁺ numbers (figure 3.3B) and a concomitant increase in CD8⁺ numbers (figure 3.3C) upon FK506 treatment. Activated T-cell numbers (CD4⁺CD25⁺) were also significantly reduced in spleen, lymph node and blood with both low and high dose treatment as compared to the untreated controls (figure 3.3E). Thus, both low and high FK506 dosing led to repression of T cell activation, albeit that this effect was less pronounced after low than after high dose treatment.

The influence of low/high FK506 on Treg frequency

Immunohistochemical analysis showed that FoxP3⁺ cells were completely absent in the intima of atherosclerotic lesions of all the vessel fragments of 26 mice. FoxP3⁺ cells were detected in the adventitia, but at very low numbers (table 3.3), that did not allow statistical analysis. To examine the Treg population more in detail, single-cell suspensions of spleen, lymph node and blood were analyzed by FACs to determine the frequency of FoxP3⁺ cells within the CD3⁺ population (Figure 3.3F). High but not low FK506 treatment significantly reduced the relative FoxP3⁺ content in spleen. FoxP3 mRNA expression in spleen was not significantly influenced by FK506 treatment (figure 3.4).

To examine if FK506 in vitro treatment might influence the viability of T cells, splenocyte viability was quantitatively measured. The number of viable cells after 72 hours of FK506 in vitro treatment was not different compared with control group (figure 3.5).

Effects of low/high dose FK506 treatment on NFκβ and NFAT signaling

The effect of low/high FK506 dosing on NFκβ activity in vivo was measured by quantitative real time PCR of IκB-α and NFκB-responsive genes such as ICAM-1 and P-selectin¹¹ in spleen. Both the low and high dose of FK506 did not alter Iκβ-α expression (figure 3.6A), nor did it change the expression of P-selectin and ICAM-1 (figure 3.6B and C). The ratio of NFATC1 to NFATC2 has previously been shown to be instrumental in T-cell differentiation and polarization¹². To investigate whether low and high dose FK506 differentially regulated NFATC1 and NFATC2 activity and thus Th cell polarization and T-cell-derived cytokine production we examined nuclear expression of NFATC1 and NFATC2 in FK506 treated Jurkat T cells by western blot as shown in Figure 3.7A. Densitometry analysis of NFATC1, NFATC2 protein bands

Figure 3.3 Flow-cytometric analysis of the effect of low and high dose FK506-treatment on % CD3+ T cells (A), % CD4+ of CD3+ T cells (B), % CD8+ of CD3+ T cells (C), CD4+ to CD8+ ratio (D), CD4+CD25+ T cells of total cells (E), and % FoxP3 of CD3+ T cells (F) in spleen, lymph node and blood. *p< 0.05.

Table 3.3. Immunohistochemistry staining of FoxP3

Treatment group	Ratio *	Total nr of positive Foxp3 cells in adventitia	Total sections checked	Nr of Foxp3 positive cells per mouse	Nr of Foxp3 positive cells per section
Control	0/9	0	45	0	0
Low	2/8	7	26	0.875	0.27
High	2/9	2	44	0.2	0.05

* Number of mice positive of FoxP3 in adventitia divided by the total number of mice

Figure 3.4 FoxP3 mRNA expression in spleen of mice that received 4 weeks FK506 treatment was measured by Q-PCR (normalization for cyclophilin).

Figure 3.5 Effects of FK506 treatment on cell viability. Splenocytes isolated from apoE^{-/-} mice were cultured under the same conditions as CD4⁺ T cells. Cells were stimulated with FK506 ranging from 0.2ng/ml to 20μg/ml for 72 hours. Viable cells were counted using trypan blue.

Figure 3.6 Iκβ (A), P-selectin (B) and ICAM 1 (C) mRNA expression in spleens of mice that received 4 weeks FK506 treatment was measured by Q-PCR (normalization for cyclophilin).

relative to that of TATA-binding protein showed that nuclear expression of NFATC2 in Jurkat cells was significantly reduced at low as well as high FK506 concentrations (Figure 3.7B). Nuclear NFATC1 expression was similarly affected by FK506 exposure, albeit that this effect did not reach significance (figure 3.7C). Furthermore, low and high FK506 concentrations both did not differentially affect the NFATC1/C2 ratio in nuclear extracts of Jurkat T cells (figure 3.7D).

Next we set out to measure IL-4, IL-10 and IFN- γ secretion patterns by CD4⁺ T cells isolated from control mice versus mice that received low and high dose treatment with FK506. Importantly a low dose of FK506 (0.05 mg/kg/day) was seen to significantly increase IL-4 to IFN- γ (figure 3.8A) and IL-10 to IFN- γ ratios (figure 3.8B), whereas high dose FK506 (1 mg/kg/day) significantly reduced the IL-10 to IFN- γ ratio compared with the PBS treatment group (figure 3.8B). Incubation of CD4⁺ T cells with low and high concentrations of FK506 in vitro reduced both IL-10 and IFN- γ production by CD4⁺ T cells (data not shown). However, consistent with the increased IL-10/IFN- γ ratio observed after in vivo treatment, low FK506 concentrations dramatically increased the IL-10/IFN- γ ratio, while high FK506 concentrations were not effective (Figure 3.8C). In the next experiment, we used Jurkat T cells to verify our observations in CD4⁺ T cells. IL-10 and IFN- γ secretion were significantly suppressed by both 20 ng/ml and 2000 ng/ml FK506 (figure 3.8D and E). Interestingly, high concentrations of FK506 (2000 ng/ml), even led to upregulation of IFN- γ secretion (figure 3.8E). In agreement with our earlier findings in CD4⁺ T cells, the IL-10/IFN- γ ratio was dramatically up-regulated at low concentrations of FK506 and normalize to base levels at higher concentrations of FK506 (Figure 3.8F).

To assess the general validity of our findings, we extended the study to THP-1 macrophages. IFN- γ and IL-10 secretion was analyzed in PMA-stimulated THP1 cells. As shown in figure 3.9, IL-10 secretion was significantly increased by 20 ng/ml of FK506, whereas 2000 ng/ml did not influence IL-10 secretion. IFN- γ secretion in the supernatant was not detectable (data not shown). The latter is consistent with previous studies that macrophages do not release significant amount of IFN- γ with LPS stimulation^{13, 14}.

Discussion

Inflammation plays an important role in atherosclerosis formation, rendering immunosuppressive therapy an attractive therapeutic modality in the treatment of this disease. Though the immunosuppressive drug FK506 is widely used in clinical transplantation, diverse side effects of this drug in clinical dosage limit its utility in other applications such as the promising treatment of (auto)immune diseases and atherosclerosis¹⁵. We have in this study investigated the dose-dependent effect of

Figure 3.7 NFATC1/NFATC2 nuclear expression. Western blot analysis of nuclear extracts from untreated Jurkat T cells or cells treated with low and high dose of FK506 (loading 30 ug protein/lane; n=6 in each group). Western blots were probed with anti-NFATC1 and anti-NFATC2. Probing with anti-TATA binding protein was used as nuclear loading

control. Representative western blot was shown in A. Signal intensities of NFATC1, NFATC2 and TATA binding protein were determined by densitometry analysis. Nuclear NFATC2 and NFATC1 expression relative to that of TATA binding protein is shown in (B) and (C), respectively. The ratio of NFATC1/NFATC2 was determined on the basis of data from (B) and (C) and is shown in panel (D). Values represent mean values $\pm$ S.E.M; *p< 0.05.

FK506 on collar-induced carotid atherogenesis in apoE^{-/-} mice. This is an extension of our previous study¹ in which only the low dose regime was applied in the treatment of atherosclerosis. Consistent with our previous study, low dose of FK506 markedly reduced atherosclerosis. High dose treatment on the other hand did not protect against atherosclerosis at all. Plaque morphometry and morphology were not influenced by FK506 treatment regardless of the dosis. Mice weight and kidney morphology were not affected by low or high dose treatment either. FACS analysis showed that both low and high FK506 treatment significantly reduced systemic CD3⁺ and CD4⁺CD25⁺ T cell

Figure 3.8 (A and B) Effects of FK506 on IL-4:IFN- γ ratio (A) and IL-10:IFN- γ ratio (B) secreted by CD4+ T cells isolated from splenocytes of mice treated with 0.05mg/kg/day and 1 mg/kg/day of FK506 and control mice. Isolated CD4+ T cells were stimulated with PMA (4ng/ml for analyzing IL-4 and IFN- γ ; 1ng/ml for analyzing IL-10 and IFN- γ) and Ionomycin (500nM). Cytokine concentration in the cell culture supernatants was determined by ELISA. 4 mice per group, n=12-14 wells in each group. (C) Effects of FK506 on IL-10/IFN- γ ratio of CD4 T cells isolated from splenocytes of 3 apoE^{-/-} mice. Isolated CD4+ T cells were pretreated with FK506 of 0, 20 ng/ml and 2000 ng/ml for 1 hour before stimulated with PMA(16ng/ml) and Ionomycin (2000nM). Cytokine in the cell culture supernatants was determined by

ELISA. n=8 per group. Effects of FK506 on IL-10 (D) and IFN- γ (E) of Jurkat T cells. Jurkat T cells were pretreated with FK506 of 0, 20, and 2000 ng/ml for 1 hour. Cells were then stimulated with PMA (8ng/ml) and 1000nM ionomycin. Cytokine in the cell culture supernatants was harvested after 48 h. ELISA was used to determine cytokine level. (F) Effects of FK506 on IL-10/IFN- γ ratio of Jurkat T cells. Values represent mean values $\pm$ S.E.M; n=6 per group; *p< 0.05.

Figure 3.9 Effects of FK506 treatment on IL-10 production by PMA stimulated THP1 cells. Cells were treated with FK506 at a dose of 0, 20 and 2000ng/ml for 32 hours and LPS of 25ng/ml for 8 hours (n=5). LPS was added 24 hr after FK506 treatment. Supernatants were harvested to analyze human IL-10 concentration by ELISA. Values represent mean values $\pm$ S.E.M; single asterisk, *p< 0.05

population.

In a subsequent experiment, it was shown that both the low and high dose of FK506 did neither change splenic gene expression of I κ B nor that of NF κ B responsive genes (P-selectin and ICAM-1). Therefore, we conclude that the differential effects of the low and high dose of FK506 are not attributable to differential regulation of NF κ B signaling in lymphatic tissue.

Wu et al recently showed that NFAT cooperates with the Treg specific transcription factor FoxP3 to control Treg suppressive function¹⁶. Therefore, FK506 treatment might indirectly influence Treg function by preventing NFAT to interact with FoxP3. Our FACs analysis showed that the high dose but not the low dose of FK506 treatment significantly reduced the percentage of FoxP3⁺ CD3⁺ T cells in spleen. Thus, besides reducing CD3⁺, CD4⁺, and CD4⁺/CD25⁺ T cell counts, high dose FK506 treatment also suppressed Treg frequency, whereas low dose treatment did not influence Treg frequency. Different effects of the low and high dose treatment on Treg numbers may suggest a dose-dependent difference in Treg response. This was confirmed in subsequent in vitro studies, which demonstrated that low dose FK506 shifted the CD4⁺ T cell balance towards a Th2 phenotype characterized by an anti-inflammatory cytokine profile, while the high dose regime favored a Th1-type cytokine response. It should be noted that FK506 only subtly influenced the balance rather than completely polarizing

towards a Th1 or Th2 response, which could lead to de-regulated Th1 or Th2 immune responses¹⁷.

Given the recent data on the interaction between NFAT and regulatory T-cell activity¹⁶, we investigated whether NFAT can act as regulator of the Th1/Th2 balance. NFAT proteins are expressed in most cells of the immune system where it plays a pivotal role in the transcription of cytokine genes and other genes critical for the immune response. Among NFAT family members, NFATC1 and NFATC2 appear to be the major isoforms relevant to T cell activation. NFATC2 and NFATC1 are critically involved in T helper cell differentiation and regulation of Th1 and Th2 cytokine gene transcription¹². An increased nuclear localization of NFATC2 correlated with poor Th2 and robust Th1 differentiation, whereas low levels of NFATC2 nuclear localization promoted Th2 differentiation^{12, 18}. Thus, the NFATC1/NFATC2 ratio has been used as a measure of Th cell differentiation, where a relative decrease in NFATC2 nuclear localization correlated with an increase in Th2 cytokine transcription, and vice versa¹². We demonstrate here that low/high doses of FK506 markedly suppresses nuclear NFATC2 and to a lesser extent also NFATC1 expression. FK506 did however not differentially affect nuclear NFATC1/C2 ratios depending on its dosing. Therefore, the observed Th2 shifted cytokine response with low dose FK506 treatment is not likely attributable to an altered balance in NFATC1/C2 transcriptional activity.

Low dose treatment of FK506 appears to induce a subtle shift towards an atheroprotective immune response. Atherosclerosis is generally thought to be a Th1 dominated inflammatory disorder. A low dose of FK506 might slightly tilt the balance towards a Th2 immune response by virtue of its ability to fine-tune Th2 type immune responses which could in turn result in the reduction of atherosclerosis observed in this study. Although, high dose FK506 treatment vice versa has been reported to aggravate disease progression⁵, we did not observe such trend in our study as IL-4/IFN- γ ratios did not differ from that of untreated controls.

The effects of FK506 on cytokine production have been intensively studied. It is well known that FK506 can suppress cytokine secretion and especially Th1 related cytokines such as IFN- γ and IL-2¹⁹. Direct effects of FK506 on Th2 mediated cytokine production or the Th1: Th2 cytokine balance however, have not been thoroughly investigated. A single study reported that FK506 was able to induce Th-2 promoting IL-4 production by dendritic cells²⁰. Moreover, a correlation was observed between FK506 treatment and the development of food allergy in post-transplant patients²¹, indicative of a Th2 switch upon FK506 treatment. Consistent with the above observations of a direct role of FK506 in Th2 activity, we found in CD4⁺ T cells, Jurkat T cells and THP1 cells that low dose FK506 (0.05 mg/kg/day) enhanced the Th2:Th1 ratio while high dose treatment reversed this effect.

Besides FK506, other immunosuppressive drugs, for instance mycophenolate mofetil (MMF), sirolimus, cyclosporine A and their concomitant anti-inflammatory effects have been studied in atherosclerotic animal models²². MMF and sirolimus showed a consistent atheroprotective potential. Regarding cyclosporine A and FK506, results in animal studies are rather contradictory, probably due to specific animal models used and large-differences in dosages²². As we show, the dosage could very well be of vital importance as many of the side-effects of FK506 and cyclosporine A reported in patients occur at high-dose and long-term regimes. Indeed our animal study indicates that only low dose suppressed atherosclerosis, whereas high dose did not. The fine-tuning effect of FK506 on the Th1/Th2 balance renders it a potential therapy for long-term treatment of chronic-inflammatory disease such as atherosclerosis.

In summary, low and high dose of FK506 differently affect atherogenesis. We show that this differential effect of low versus high dose FK506 unlikely involves the NF κ B signaling cascade in lymphatic tissue. Our findings instead indicate that low dose FK506 protects against atherosclerosis by skewing T-cells towards a Th2 immune response, whereas the high dose did not. The fact that low dose treatment of FK506 only induces a mild shift in Th1/Th2 balance also implies that it might be very effective for long-term therapy in Th1 dominated immune disorders such as atherosclerosis.

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4

Low-dose FK506 treatment does not affect murine aneurysm formation

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Abstract

The immunosuppressive drug FK506 (tacrolimus), is widely used in patients following transplantation. In a previous study, we showed that low-dose FK506 treatment inhibited the initiation of collar-induced atherosclerosis in apoE^{-/-} mice. An abdominal aortic aneurysm (AAA) is a permanent dilation of the arterial wall of the abdominal aorta. Inflammatory processes play a significant role in this vascular pathology. Given the inflammatory feature of AAA and potential immunosuppressive effect of low-dose FK506, we hypothesized that low-dose FK506 treatment would decrease AAA formation. To test this hypothesis, we investigated the effect of low-dose FK506 treatment in angiotensin II (Ang II)-induced aneurysm formation in the abdominal aorta of apoE^{-/-} mice.

Contrary to our expectations, FK506 treatment did not protect against murine AAA formation. The distinct pathological characteristics of atherosclerosis and aneurysm formation might explain the differential effect of low-dose FK506 in these vascular pathologies.

Introduction

AAA is a permanent dilation of the arterial wall¹. Degradation of elastin and collagen weakens the aortic wall and allows the aneurysm to develop.

A history of atherosclerotic disease has been considered as the primary cause of AAA². One of the most affected site by the atherosclerosis is the infrarenal abdominal aorta, which is also the most common site of AAA². In addition to atherosclerosis as a possible predisposing factor for aneurysm formation, several other factors are thought to be involved, including genetic predisposition, inflammation, hemodynamic factors and structural remodeling of the extracellular matrix (ECM)^{2,3}.

The role of inflammatory cells in AAA formation has been studied intensively. Treska et al showed that higher expression of IL-6, IL-8 and TNF- α were found in the walls of ruptured aneurysms compared to non-ruptured aneurysms⁴. Many cells within these inflammatory infiltrates were stained positive for the leukocyte common antigen, CD45⁴. Satta et al showed that the inflammatory infiltrate was largely mononuclear and located at the media and adventitia junction of human AAA. Immunophenotypic analysis of the inflammatory cells indicated the presence of T cells, B cells and macrophages in variable numbers and only occasional neutrophils. Furthermore, the presence of elastin degradation was associated with increased mononuclear inflammatory cell infiltrates⁵. Among mononuclear cells, T cells may play a significant role in AAA formation given the observation that human aneurysm tissue displayed substantial presence of identical β -chain T cell receptor transcripts⁶.

In our previous study, low-dose FK506 treatment inhibited the initiation and progression of collar-induced atherosclerosis in apoE^{-/-} mice⁷. FK506, a macrolide antibiotic with major immunosuppressive properties, is used to prevent rejection after organ transplantation⁸. At a molecular level, FK506 enters the cytoplasm and binds to the intracellular protein FK506 binding protein (FKBP)⁹. This complex binds and blocks calcineurin, a Ca²⁺-calmodulin-regulated protein phosphatase. As a consequence, calcineurin fails to dephosphorylate the cytoplasmic component of NFAT, thereby inhibiting transport of NFAT to the nucleus and NFAT-AP1 function in transactivating cytokine genes, genes that regulate T cell proliferation and other genes that orchestrate an active immune response.

Given the inflammatory features of AAA and the immunosuppressive function of FK506, we hypothesized that FK506 treatment would protect against AAA. To test this hypothesis, a murine model of Ang II-induced AAA was used to study the effect of

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FK506 treatment on aneurysm formation. Surprisingly, our data showed that low-dose FK506 treatment did not reduce AAA formation.

Materials and Methods

Animals

Male apoE^{-/-} mice at 10-12 weeks of age on a C57Bl6 background were obtained from Charles River (Maastricht, Netherlands). Animals were maintained in accordance with the Dutch government guidelines and animal experiments were approved by the regulatory authority of the University of Maastricht. Mice were fed a normal chow diet throughout the experiment.

Chronic Ang II infusion to induce aneurysm formation and FK506 treatment

Aneurysm formation was induced by subcutaneous Ang II infusion¹⁰. Alzet mini-osmotic pumps (Alzet type 2004) containing Ang II dissolved in phosphate buffered saline (PBS) (10 mM sodium phosphate, 150 mM sodium chloride; pH=7.4) were implanted into apoE^{-/-} mice (n=20) to result in the subcutaneous delivery of 1 ng/g/min of Ang II for 28 days. To study the effect of FK506 on Ang II-induced aneurysm formation, pumps were filled with equal volume of Ang II and FK506 dissolved in PBS in apoE^{-/-} mice (n=18) to mediate the subcutaneous delivery of 1 ng/g/min of Ang II and 0.05 mg/kg/day for 28 days. Pumps were placed subcutaneously in the neck of anesthetized mice through a small incision that was closed using a silk suture (5-0).

Tissue harvesting and histological analysis

Aortic tissue from mice which died preliminary was dissected and fixed in 1% paraformaldehyde and embedded in paraffine. After 4 weeks of Ang II infusion and FK506 treatment, mice were sacrificed and the arterial system was perfused with PBS containing 0.1 mg/ml nitroprusside (Sigma) through a catheter inserted into the left cardiac ventricle and subsequently with 1% paraformaldehyde. Aortic tissue was removed from the ascending aorta to the aortic bifurcation, fixed overnight in 1% paraformaldehyde and embedded in paraffine. Ten cross-sections (4 µm thick) were cut at 200 µm intervals throughout the abdominal aorta starting at the middle of the aneurysm. From each level, a cross-section was stained with elastica von Gieson (EvG) for analysis of aneurysm area. Morphometric analysis was performed by one blinded investigator (LB, intra-observer variability was < 10%).

Quantification of Aneurysm Formation

Aneurysm severity was analyzed according to Daugherty et al¹¹. In this scoring system, type 0 depicts no aneurysm (the suprarenal region of the aorta was not obviously

different from naive apoE^{-/-} mice without Ang II treatment; type I depicts a dilated lumen with no thrombus; type II depicts remodeled tissue often containing thrombus; type III depicts a pronounced bulbous form of type II containing thrombus; and type IV depicts multiple aneurysms containing thrombus. To analyze this measurement semiquantitatively, the numerical score allocated to the type of aneurysm for each mouse in a group was averaged to generate a pathology score for statistical comparison¹².

Statistical analysis

Values are expressed as mean $\pm$ SEM and a Mann-Whitney non-parametrical test was used to compare individual groups of animals. Probability values of < 0.05 were considered significant.

Results

FK506 treatment did not protect against Ang II-induced aneurysm formation

Infusion of Ang II for 4 weeks induced AAA formation in 60% (12 out of 20, n=20 mice) of the mice without FK506 treatment and 44% (8 out of 18, n=18 mice) of mice with FK506 treatment. No aneurysm formation was found in 8 control and 10 FK506-treated mice.

The death of mice was caused by the rupture of the aneurysm. Fatal ruptures (death caused by rupture of aneurysm) were found in 15% of the control group (3 out of 20). Comparable numbers were found in FK506-treated mice (22%, 4 out of 18). The occurrence of non-fatal rupture was also similar in control versus FK506-treated mice, occurring in 45% (9 out of 20) of the control group and 22% (4 out of 18) of the FK506 treated mice (figure 4A). The majority of AAAs in both groups were characterized as type III (figure 4B)¹¹. FK506 treatment also did not change the pathological score of the AAA (figure 4C). There was no difference between control and FK506-treated mice in the abdominal aneurysm area and lumen area as shown in figure 4D.

Discussion

In our previous study, inhibition of calcineurin signaling pathway by FK506 at a low dose resulted in an athero-protective effect. In addition, the low dose of FK506 was able to suppress systemic T cell numbers in vivo and cytokine production in vitro. This triggered us to further investigate the effect of FK506 on aneurysm formation as inflammatory processes play a significant role in this vascular pathology. In contrast to our expectations, low dose FK506 treatment did not protect against AAA formation.

The distinct pathological characteristics of atherosclerosis and aneurysm formation might explain the different effect of low dose FK506 in these vascular pathologies. Thus

Figure 4 Low dose FK506 treatment did not protect against AAA formation. FK506 treatment did not affect incidences of fatal and non-fatal rupture (A). FK506 treatment did not change the severity of aneurysm (B) or the average pathological score (C). In addition, FK506 did not affect aneurysm and lumen size (D).

although aneurysms occur in the context of atherosclerosis¹³, the majority of patients with advanced atherosclerosis do not develop an aneurysm^{13, 14}. Conversely, patients that have no signs of atherosclerotic disease are presented with aneurysm formation¹⁴. Mechanisms involving a balance in cytokine secretion have been put forward to explain the differences between these two vascular pathologies. A number of studies clearly indicated that human aneurysmal tissue was characterized by the predominance of Th2 cytokines and a lack of Th1 cytokines^{15, 16, 17}, although there are also reports showing elevated Th1 cytokines in patients with aneurysms^{18, 19}. Shimizu et al proposed that aneurysm formation required initial inflammatory cell recruitment which is characterized as a shift to Th1 response. However, when the local environment is skewed toward a Th2 reaction, aneurysms would progress¹⁴. As shown in chapter 3, low-dose FK506 treatment was able to induce a moderate Th2 skewing by increasing the IL-4/IFN- γ and IL-10/IFN- γ ratio in CD4⁺ T cells, Jurkat T cells and PMA-differentiated THP1 cells. Thus, low-dose FK506 treatment resulted in skewing to a mild Th2 response, which might theoretically promote AAA progression. This, however, was not observed in our

study. We hypothesize that the differences in T cell polarization in either atherosclerosis or aneurysm formation may underlie the differential effect of low dose FK506 treatment in these two vascular pathologies. This, however needs further study.

Many different immune cells have been linked to the initiation and the development of AAA²⁰. In addition to macrophages, T and B cells have been shown to be present in human and mouse AAA lesions²⁰. In addition, a significant increase in mast cell numbers was observed in adventitia in AAA patients compared with control groups²¹. Furthermore, treating Ang II-infused apoE^{-/-} mice with Tranilast (a mast cell degranulation inhibitor) significantly reduced the dilatation of the aorta and the inflammatory response compared with the untreated group²¹. FK506 inhibited mast cell cytokine production^{22, 23} and inhibited cytokine secretion of IL-2, IL-3, IL-4 and granulocyte-macrophage colony stimulating factor by MC/9 cells (the mast cell clone)²². Furthermore, FK506 inhibited histamine release by peritoneal mast cells more potently compared with clinically used steroids²³. Protease release by mast cells are thought to be involved in the pathogenesis of AAA²⁰. However, the effect of FK506 on mast cell protease release is not clear. It is thought that elastin degradation will critically contribute to AAA formation¹. Given the fact that a MMP inhibitor was able to significantly inhibit AAA formation²⁴, it is possible that inhibition of proteolytic activity is more effective than cytokine inhibition in the treatment of AAA formation.

In addition to inflammatory cells, growth factors have been intensively investigated on their roles in AAA formation (reviewed in²⁰). TGF- β has been the subject of many investigations²⁰. Firstly, heterozygous mutations in either type I or type II TGF- β receptor lead to more intense TGF- β signaling and this increased TGF- β signaling was correlated with increased incidence of AAA in patients²⁵. Secondly, murine AAA formation was associated with increased TGF- β signaling and could be prevented by either a TGF- β antagonist or an Ang II type 1 receptor (AT1) blocker²⁶. Ang II is known for its role in the initiation and propagation of AAA²⁷. In addition, Ang II induced the secretion of thrombospondin-1 in human mesangial cells²⁸ and thrombospondin-1 is a potent inducer of TGF- β activation²⁸. A link between TGF- β and FK506 has been reported, thus FK506 promoted TGF- β receptor transphosphorylation in a mink epithelial cell line²⁹. In addition, FK506 activated the TGF- β signaling by inducing Smad phosphorylation and nuclear translocation in vascular smooth muscle cells (SMC)³⁰. Based on the data provided above, FK506 treatment might even enhance AAA formation. Nevertheless, FK506 did not influence AAA in our study. Despite the above evidence showing the involvement of TGF- β in the pathogenesis of AAA, there is also literature suggesting that TGF- β could maintain ECM integrity and inhibit mast cell function (reviewed in

²⁰). On the one hand, TGF- β can promote ECM degradation³¹⁻³³, stimulate the growth of bone marrow-derived mast cells³⁴ and induce mast cell protease release³⁵⁻³⁷; on the other hand, TGF- β can stimulate collagen I³⁸ and III³⁹ synthesis by both fibroblasts and myofibroblasts, and in addition induce mast cell apoptosis⁴⁰ and inhibit mast cell maturation, all factors that could protect against AAA formation or progression⁴¹. The bipolar nature of TGF- β during AAA needs further study.

In conclusion, our study showed that low-dose FK506 did not protect against Ang II-induced AAA formation in apoE^{-/-} mice and suggest no therapeutic opportunities of low dose FK506 in the treatment of AAA.

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5

Leukocyte NFATC2 deficiency deteriorates rather than protects against atherosclerosis by facilitating memory T cell recruitment

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Abstract

Nuclear Factor of Activated T-cells C2 (NFATC2) is expressed in several leukocyte subsets and is a crucial transcriptional regulator of numerous inflammatory genes during immune responses. The recently reported role of NFATC2 in regulatory T-cell function as well as the finding that the NFAT inhibitor FK506 diminished atherosclerosis, suggestive of a pro-atherogenic role of this protein, led us to investigate the effect of leukocyte deficiency of NFATC2 on atherosclerosis.

Contrary to our expectations, NFATC2^{-/-} bone marrow transplanted LDLr^{-/-} mice showed significantly increased lesion area and progression in the aortic arch. While plaque macrophage, CD45⁺ cell and collagen content were unaltered, CD3⁺ T cell content was sharply increased in lesions of NFATC2^{-/-} chimeras. FACS analysis of T-cell subsets revealed a marked decrease in CD4/CD8 ratio and a general shift from naïve T-cells towards migratory CD44^{hi}CD62L^{low} effector/memory T-cells in NFATC2 deficiency. In concert with the latter, grafting of NFATC2^{-/-} T-cells in spleen was impaired, whereas homing to the atherosclerotic plaque was 2.7 fold increased. Moreover, NFATC2 deficient T-cells displayed enhanced CD3 expression, a mitogenic response to CD3 activation and impaired IL-10 production, which was not attributable to a change in regulatory T cell function. Collectively, our findings suggest that NFATC2 deficiency translates in augmented rather than compromised T-cell response, which may underlie the aggravated atherogenic response in NFATC2^{-/-} mice.

Introduction

The transcription factor Nuclear factor of activated T cells (NFAT) is essential for an adequate immune response¹. It is expressed in a variety of immune cells such as T and B lymphocytes, natural killer cells², monocytes³ and myeloid cells⁴. The NFAT family encompasses five well-characterized members with NFATC1 and NFATC2 as major isoforms relevant to T cell activation⁵. NFATs regulate the induction of expression of several cytokine genes, such as interleukin (IL)-2, IL-3, IL-4, IL-5, interferon (IFN)- γ , tumour necrosis factor (TNF- α), granulocyte-macrophage colony-stimulating factor, and TNF family members, such as CD40L, CTLA-4, and FasL¹. However, NFAT family members C1 and C2 were also reported to regulate T helper (Th) cell differentiation⁶ and mice deficient in NFATC2 showed modest splenomegaly with hyperproliferation of T- and B-lymphocytes and an enhanced allergic phenotype characterized by eosinophilia *in vivo* and *in vitro*^{2,7-9}. Despite the pleiotropic activity of NFAT, intervention in NFAT signaling by immunosuppressants such as FK506/tacrolimus is widely regarded as an effective therapeutic strategy in a number of chronic inflammatory and autoimmune disorders as well as in transplant rejection¹⁰⁻¹². Several lines of evidence indicate that NFAT is implicated in chronic inflammatory processes that underlie atherosclerosis as well. Firstly low dose treatment with FK506 inhibited the initiation and progression of collar-induced atherosclerosis in apoE^{-/-} mice¹³, although the actual molecular NFAT target mediating this effect remains to be identified. Secondly, NFATC2 was recently reported to control regulatory T-cell function and its deficiency led to a Th2 biased T-cell response^{2,14,15}, both processes that were shown to attenuate the atherogenic response. However, no data is available on its role in atherosclerosis. On the basis of these findings, it is conceivable that NFATC2 deficiency in hematopoietic lineages will protect against atherosclerosis. Here, we show that, contrary to our hypothesis, leukocyte NFATC2 deficiency deteriorated atherosclerosis. NFATC2 deficiency led to augmented rather than compromised immune responses. NFATC2 deficient T cells showed an enhanced proliferative activity and displayed a memory/activated T cell phenotype more prone to migrate to the atherosclerotic plaque, which may have contributed to the exacerbation of atherosclerosis.

Materials and Methods

Animals

Female LDLr^{-/-} mice on a C57Bl6/J background (6- to 8-weeks-old) were obtained from Charles River (Maastricht, the Netherlands). The generation of NFATC2 deficient mice has been described previously⁷. In short, NFATC2 deficient mice were backcrossed seven generations onto the C57Bl6/Sv129 background. Animal experiments were

approved by the regulatory authority of the Maastricht University and were performed in compliance with the Dutch government guidelines.

Irradiation and bone marrow transplantation (BMT)

One day before the transplantation, LDLr^{-/-} recipient mice were exposed to a single dose of 9 Gy of total body irradiation (Rontgen source). Bone marrow was harvested by flushing femurs and tibias of either WT or NFATC2^{-/-} donor mice with phosphate-buffered saline (PBS). Single cell suspensions were prepared by passing the cells firstly through an 19-gauge needle and then a 25 gauge-needle. Irradiated recipients received 5×10^6 bone marrow cells by injection into the tail vein. BMT-recipient mice were housed in sterilized filter-top cages and fed a sterilized normal chow diet and sterile drinking water containing neomycin (100 mg/L) and polymyxin B (60,000 U/L). Four weeks after BMT, drinking water was switched to normal water and the diet was switched to a high-cholesterol diet (1.25% cholesterol, D12108, Research Diet) for another 23 weeks to induce atherosclerosis.

Characterisation of chimerism and serum lipid levels of LDLr^{-/-} mice after BMT

Determination of the chimerism in transplanted mice was performed as described previously¹⁶. Genomic DNA isolated from the blood of recipient LDLr^{-/-} mice, at 27 weeks after BMT using the Illustra blood genomic prep Mini spin kit (GE Healthcare). The standard curve was composed of DNA from LDLr^{-/-} and LDLrWT blood mixed at different ratios. Chimerism was determined by quantifying the amount of LDLrWT DNA in samples from 40 µl peripheral blood. Samples were assayed in duplicate using the Taqman IQ™ SYBR Green Super Mix (BIO-RAD). Chimerism was calculated as the percentage of LDLrWT DNA in the blood samples. Serum cholesterol levels were determined with the CHOD-PAP method (Roche Diagnostics).

Histological analysis of aortic arch for atherosclerosis

Mice were sacrificed at 27 weeks after BMT (4 weeks on regular chow diet followed by 23 weeks on high-cholesterol diet). To study the development of atherosclerosis, sections of the aortic arch were prepared and analyzed as described previously¹⁷. Briefly, the arterial system was perfused with PBS containing 0.1 mg/ml nitroprusside (Sigma). The aortic arch, including its main branch points, was excised and fixed with 1% paraformaldehyde. Forty cross-sections (4 µm thick) from the longitudinally imbedded aortic arch were cut. A series of twenty sections, which represented the complete arch and branch points, were analyzed. Total plaque burden is defined as the summed area of all aortic arch lesions. Lesions containing only foam cells were categorized as early whereas advanced lesions were characterized by the presence of necrotic core and/or fibrous cap. The relative collagen area in atherosclerotic lesion that was stained

positive for Sirius red, was determined under a microscope coupled to a computerized morphometry system (Quantimet 570, Leica). Morphometric analysis was performed by one blinded investigator (LBai; intra-observer variability was < 10%).

Immunohistochemical stainings

Sections were stained with the following antibodies: Rabbit anti-mouse/human CD3+ (1:200, Dako) to detect CD3+ T cells, Mac3 rat monoclonal antibody (1:30, BD Biosciences Pharmingen) to detect macrophages, CD45 (1:5000, BD Biosciences Pharmingen) to detect leukocytes, and cleaved caspase-3 (1:100, Cell Signalling) to detect apoptotic cells. To determine the relative amounts of macrophages, leukocytes and cleaved caspase-3-positive cells in the lesions, the number of Mac3, CD45 and cleaved caspase-3-positive cells in the lesions were divided by the corresponding lesion area. In addition, the relative CD3+ T-cell content was calculated by dividing the number of CD3+ positive cells by the total number of cells in the lesion or by the corresponding lesion size. All measurements were conducted by one investigator (LBai, intra-observer variability was < 10%).

Quantitative RT-PCR

RNA was extracted from the spleen lysate with Nucleospin RNA II kit (MACHEREY-NAGEL). cDNA was generated with iScript™ CDNA synthesis kit (BIO-RAD). Real-time PCR was done with a Taqman IQ™ SYBR Green Super Mix (BIO-RAD). Primer sequences of CXCR3, CCR3, CCR4, CCR6, CCR7, Foxp3, 18S and cyclophilin were shown in table 5.1. An average expression of 18S and cyclophilin was considered reference value for total RNA expression.

Table 5.1

	<i>Forward primer (5'-3')</i>	<i>Reverse primer (5'-3')</i>
CXCR3	TGCTAGATGCCTCGGACTTT	CGCTGACTCAGTAGCACAGC
CCR3	TGGCATTCAACACAGATGAAA	TGACCCCAGCTCTTTGATTC
CCR4	GGTCTTCCTGCCTCCTCTCT	GGACAGGACGAACAGCAAAT
CCR5	GCCAGAGGAGGTGAGACATC	CCAAAGATGAATACCAGGGAG
CCR7	GTGGTGGCTCTCCTTGTCAT	GAAGCACACCGACTCGTACA
Foxp3	CCAGTCTGGAATGGGTGTCC	CTGCTTGGCAGTGCTTGAGA
Cyclophilin	CAAATGCTGGACCAAACACAA	TTCACCTTCCCAAAGACCACAT
18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG

Cytokine measurements

Serum levels of IL-6, IL-10, IL-12, and IFN- γ were measured using Cytometric Bead Array (552364, BD Biosciences) according to the manufacturer's instructions.

Splenocytes isolation and cytokine analysis

Spleens from donor NFATC2^{-/-} and WT mice (n=5 mice in each group) were forced through mesh screens to yield a single-cell suspension in splenocyte medium (RPMI medium supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin (Gibco), and 10% FCS). Erythrocytes were lysed by erythrocyte lysis buffer (0.15 M NH₄Cl, 10 mM NaHCO₃ and 0.1 mM EDTA; pH=7.4) for 5 minutes. Thereafter, cells were washed and resuspended in splenocyte medium. Splenocytes were seeded in 96-well plates at a density of 5×10^6 cells/ml. Cells were incubated for 48 hours with 1 ng/ml PMA (Sigma) and 500 nM Ionomycin (Sigma) (n=9 mice per group). Supernatants were harvested and stored at -80°C before analysis. Murine IFN- γ and IL-10 were assayed by ELISA using antibodies according to the manufacturer's instructions (BD Bioscience).

Fluorescence-activated cell sorting (FACS)-analysis

Splenocytes and blood were isolated from the BMT recipient NFATC2^{-/-} and WT mice for FACS analysis. Splenocytes were isolated and used to make single-cell suspensions. Erythrocytes in peripheral blood and spleen were removed by hypotonic lysis with NH₄Cl. Cells were incubated first with anti-CD16/32 (eBioscience) to block Fc binding and stained with anti-CD3-FITC, anti-CD8-Pacific blue, anti-CD4-Pacific blue (eBioscience), anti-CD25-APC (eBioscience) and anti-CD45R/B220-PerCP (BD-Biosciences Pharmingen). Foxp3-positive cells were detected with PE anti-mouse/rat Foxp3 Staining Set, according to the manufacturer's instruction (eBioscience). Splenocytes and peripheral blood leukocytes were further incubated with anti-CD62L-PE Cy7 (eBioscience) and anti-CD44-PE (BD-Biosciences Pharmingen) to identify naïve T cells (defined as CD44^{low}CD62L^{high}) and memory/effector T cells (defined as CD44^{high}CD62L⁺).

In vivo trafficking experiments

WT and NFATC2^{-/-} mice received an intravenous injection of 90ug LPS. Two hours after LPS injection, splenocytes from WT and NFATC2^{-/-} mice were isolated and labelled with 2 μ M CFSE (Invitrogen) in PBS at 37°C for 15 minutes. Labelled cells were then incubated in RPMI medium at 37°C for 30 minutes to ensure that by-products had passively diffused into the extracellular medium. After washing with PBS containing

0.1% BSA, 3×10^7 of labelled cells in 0.2 ml of PBS were injected intravenously into apoE^{-/-} recipient mice (37- 40 weeks old, 4 received labelled splenocytes from WT mice and 5 received labelled splenocytes from NFATC2^{-/-} mice). Forty two hours after the injection, mice were sacrificed and perfused with PBS. Spleen and aortic arch were harvested to identify the amount of CFSE⁺ positive cells that migrated to spleen and atherosclerotic lesions. The percentage of CFSE-labeled splenocytes was determined by flow cytometry equipped with a 488 nm excitation source.

Perfused aortic arches were placed in Tissue-Tek O.C.T. embedding medium (Miles Inc), snap-frozen at -160°C in liquid nitrogen-cooled isopentane (Baxter Scientific). Twenty cross-sections (4µm thick) from the longitudinally imbedded fresh-frozen aortic arches were sectioned. Four sections with a distance of 20 µm each were selected to analyze CFSE⁺ cells in the lesions. Nuclei were counterstained using Mounting Medium with DAPI (VECTOR). Overlay images were acquired using fluorescence microscope (Leica DM 5000B) with the filter system I3 to detect CFSE⁺ cells, and SGR to detect DAPI. Lesional CFSE⁺ cell content was calculated from the total number of CSFE⁺ cells divided by that of all cells in the lesion (n=57 lesions for WT and n=74 lesions for NFATC2^{-/-}). Analysis was performed by one blinded investigator (LBai; intra-observer variability was < 10%).

T cell proliferation and regulatory T cell function assays

Cell proliferation experiment and regulatory T cell function assay were performed as described before¹⁸. Cells were purified and pooled from 3 mice per group at the time of sacrifice. CD4⁺CD25⁻ cells (0.5×10^5 cells) were cultured alone or in co-culture with CD4⁺CD25⁺ cells at diverse concentrations (0.5×10^5 , 0.25×10^5 , 0.12×10^5 , 0.06×10^5), in flat-bottomed 96-well microplates (total volume 200 µL/well). Cells were stimulated with purified soluble CD3-specific antibody (1 µg/mL, Pharmingen) in the presence of antigen-presenting cells (0.1×10^5 cells) purified on CD11c-coated magnetic beads (Miltenyi Biotech). Cells were cultured at 37°C for 72 hours, pulsed with 1 µCi of (³H) thymidine (Amersham) and incubated for another 18 hour. Thymidine incorporation was assessed using a TopCount NXT scintillation counter (Perkin Elmer).

Statistics

Statistical analyses were performed using a nonparametric Mann-Whitney *U* test. The plaque area, plaque composition and the number of CFSE⁺ positive cells in the lesion in the in vivo traffic experiment was compared with independent sample T test. Data are expressed as mean ± SEM. Probability values of < 0.05 were considered significant.

Results

Characterization of NFATC2^{-/-} vs WT bone marrow chimeras

At 27 weeks after bone marrow transplantation, blood was collected to determine chimerism in white blood cells by quantitative real-time PCR. An average of 96.8% of the white blood cells was of donor origin, establishing successful engraftment. The degree of chimerism was identical in NFATC2^{-/-} and WT transplanted mice (97.1 ± 0.3 vs 96.5 ± 0.5 %). Serum cholesterol levels were not different between the two groups

Figure 5.1 Effect of leukocyte NFATC2 deficiency on serum total cholesterol level.

(31.3 ± 2.0 mmol/L vs 27.2 ± 2.6 mmol/L, figure 5.1).

Hematopoietic deletion of NFATC2 increased atherosclerosis in LDLr^{-/-} mice

Although NFATC2 deficiency might influence inflammatory status of non-atherosclerotic vessels, in this study, we focus on the effect of NFATC2 on atherosclerotic lesions. We analyzed 170 aortic arch lesions of the control mice ($n=9$) and 197 lesions of NFATC2^{-/-} transplanted mice ($n=8$). Total plaque burden did not differ between the two groups (Figure 5.2A). However, significantly more plaques of NFATC2^{-/-} transplanted mice had progressed to an advanced stage (88% for WT vs 95% for NFATC2^{-/-}, $p=0.02$, Figure 5.2B) and less plaques of NFATC2^{-/-} transplanted mice were retained in the early stage of lesion development (12% for WT vs 5% for NFATC2, $p=0.02$, figure 5.2C). Average advanced plaque size was significantly increased in NFATC2^{-/-} transplanted mice compared to WT control ($1.38 \pm 0.10 \mu\text{m}^2 \times 10^5$ for control vs $1.75 \pm 0.16 \mu\text{m}^2 \times 10^5$ for NFATC2^{-/-}, $p=0.049$, Figure 5.2D). There was no difference in average initial lesion size between the two groups ((Figure 5.2E, $p=0.917$) possibly indicating that NFATC2 involvement was predominant in advanced plaque development. In particular, plaque burden at the subclavia and arcus aortae of NFATC2^{-/-} transplanted mice was increased, as compared to that of controls (subclavia: $3.57 \pm 0.66 \mu\text{m}^2 \times 10^4$ for control vs $5.48 \pm 0.64 \mu\text{m}^2 \times 10^4$ for NFATC2^{-/-}, $p=0.031$; arcus aortae: $1.74 \pm 0.19 \mu\text{m}^2 \times 10^5$ for control vs $3.41 \pm 0.44 \mu\text{m}^2 \times 10^5$ for NFATC2^{-/-}, $p=0.001$, Figure 5.2F and G). Thus, deletion of NFATC2 in hematopoietic cells resulted in an increase in advanced atherosclerotic lesion formation. Representative HE stained cross sections of aortic arch were shown in figure 5.2H. Full color figures of HE stained cross-sections are shown on page 172.

Figure 5.2 The absence of leukocyte NFATC2 did not affect total plaque burden (A). Leukocyte NFATC2 deficiency resulted in an increase in the percentage of advanced lesion (B) and decrease in the percentage of initial lesion (C) relative to total lesions. Average advanced plaque area was significantly increased in NFATC2^{-/-} transplanted mice compared to control (D). However, average initial lesion size was not different between the two groups (E). NFATC2 deficiency increased lesion size in the artery subclavia (F) and the arcus aortae (G). H shows representative HE stained cross-sections of control versus NFATC2^{-/-} transplanted mice, respectively. Full color figures of HE stained cross-sections are shown on page 172.

*P < 0.05, ** P<0.01 compared with WT.

As for plaque composition, the relative CD3⁺ T-cell content of advanced lesions were dramatically increased in NFATC2^{-/-} transplanted mice compared with controls on plaque area (9.5 ± 2.1 and 3.6 ± 1.0 CD3⁺ T cells/ $\mu\text{m}^2 \times 10^5$, respectively; $p=0.017$, data not shown) as well as expressed as % of total cell counts (3.5 ± 1.0 % and 1.1 ± 0.3 %; $p=0.024$, Figure 5.3A). Representative pictures of CD3⁺ staining from control and NFATC2^{-/-}-transplanted mice are shown in Figure 5.3B. Full color figures are shown on page 172. CD45⁺ leukocyte (Figure 5.4A) and Mac3⁺ macrophage numbers (Figure 5.4B), lipid core size (Figure 5.4C) as well as collagen content (Figure 5.4D) did not differ between the two groups. Furthermore, the percentage of cleaved caspase-3-positive cells relative to advanced lesion area was not different between the two groups (Figure 5.4E). Interestingly, the degree of calcification, characterized by mineralization and the presence of chondrocytes was dramatically increased in NFATC2 transplanted mice compared with control (6.4% for control vs 20.1% for NFATC2^{-/-}, $p=0.0003$, Figure

Figure 5.3 Leukocyte NFATC2 deficiency significantly increased the amount of CD3-positive cells relative to total cells in advanced lesions compared with controls. Representative pictures of CD3⁺ staining of aortic arch of control and NFATC2^{-/-} transplanted mice are shown in B. Full color figures are shown on page 172. Values represent mean $\pm$ SEM, * $P < 0.05$ compared with WT.

5.4F).

Hematopoietic NFATC2 deficiency alters lymphocyte pattern in spleen and circulation

The remarkable increase in plaque T-cell content prompted further investigation of T-cell function and differentiation. Hereto single-cell suspensions of spleen, and blood were analyzed for T cell subsets by FACS (Figure 5.5 and 5.6). CD3⁺ T cell numbers in spleen and blood were not altered (Figure 5.5A), while B cell counts were unchanged in spleen but significantly increased in blood of NFATC2^{-/-} compared with WT transplanted mice (Figure 5.5A). Blood T-cells in NFATC2^{-/-} chimeras displayed a sharply reduced CD4⁺ to CD8⁺ ratio (Figure 5.5B). Despite this reduced ratio, CD3⁺ T-cells in blood were markedly enriched in CD4⁺CD25⁺ T cells in NFATC2^{-/-} transplanted mice (Figure 5.5B). Within this subset, not only the blood CD4⁺CD25⁺Foxp3⁺ regulatory

Figure 5.4 Influence of leukocyte NFATC2 deficiency on plaque composition. Leukocyte content (CD45 staining) (A) and macrophage content (Mac3 staining)(B) relative to advanced lesion area were not different between the two groups. Lipid core content (C) and collagen content (D) as measured by a Sirius red staining were not different between the two groups. Cells positive for cleaved caspase-3 relative to advanced lesion area was not different between the two groups (E). The percentage of calcification characterized by mineralization and the presence of chondrocytes relative to non-calcified lesions was increased in NFATC2^{-/-} mice compared with NFATC2^{+/+} (F). Values represent mean $\pm$ SEM, ***P < 0.001 compared with WT.

T-cells (Treg) but also the spleen CD4⁺CD25⁺Foxp3⁻ effector T cells were increased with NFATC2 deficiency (Figure 5.5C). The significant reduction in blood CD4⁺CD25⁻ among CD3⁺ T cells might account for the decreased CD4/CD8 ratio (Figure 5.5B). NFATC2^{-/-} chimeras did not display significant difference in percentage of CD4⁺, CD8⁺, CD4⁺CD25⁺ and CD4⁺CD25⁻ of CD3⁺ T cells in spleen (Figure 5.5D). Naïve T cells, defined as CD44^{low}CD62L^{high} CD4⁺ cells, were significantly reduced in blood of NFATC2^{-/-} transplanted mice, while memory/migratory CD44^{high}CD62L^{low} T cells were increased (Figure 5.6D). Of note, the CD8⁺ T cell population gave a similar pattern with a marked decrease in naïve T cells and a minor, non-significant increase in memory T cell numbers in spleen (Figure 5.6E). Focusing on the CD4⁺CD25⁺ T cell population, we observed a significant decrease in the ratio of naïve to memory T cells (Figure 5.6F). Interestingly, not only fewer T-cells were CD62L⁺, also mean CD62L expression by CD4⁺, CD8⁺ and CD4⁺CD25⁺ CD62L^{high} T-cells was substantially reduced in NFATC2^{-/-} chimeras (Figure 5.6 G, H and I, respectively).

Although CD3⁺ T cell numbers in spleen and blood were not altered, CD3⁺ mean fluorescence intensity (MFI) of CD3⁺ T-cells was markedly enhanced in NFATC2^{-/-} transplanted mice (Figure 5.7A). This may point to an increased responsiveness of NFATC2^{-/-} T lymphocytes to anti-CD3 antibody treatment. In keeping with this notion the mitogenic response of CD4⁺CD25⁻ T cells upon anti-CD3 antibody treatment *in vitro*, as measured by incorporation of [3H] thymidine was increased by a significant 30% in NFATC2^{-/-} T-cells (Figure 5.7B).

Splenocyte NFATC2 deficiency reduces spleen engraftment but enhances homing to atherosclerotic lesions

As our FACS showed a marked increase in migratory T-cell subsets and in particular central memory T cells in blood and spleen of NFATC2 deficient BMT recipients, we argued that this shift in migratory capacity may have accounted for the higher plaque T-cell content in NFATC2^{-/-} chimeras. CFSE⁺ CD4⁺ splenocytes isolated from NFATC2^{-/-} vs WT chimeras were injected into apoE^{-/-} recipients with established atherosclerotic lesions. Forty two hours after injection, CFSE⁺ cells could be detected in recipient spleens as measured by FACS. In agreement with our hypothesis, recovery of NFATC2^{-/-} CFSE⁺ cells in spleen was dramatically reduced compared to that of WT CFSE⁺ splenocytes (Figure 5.8D). In contrast, the percentage of CFSE⁺ cells of total cells in plaque was significantly higher in recipients that had received NFATC2^{-/-} CFSE⁺ splenocytes than in recipients receiving WT CFSE⁺ splenocytes (Figure 5.8E). Representative overlays of images of the aortic arch from apoE^{-/-} mice that receive WT and NFATC2^{-/-} CFSE⁺ splenocytes are shown in figure 5.8F. Full color figures

Figure 5.5 Flow-cytometric analysis of the effect of NFATC2 deficiency on CD3⁺, CD4⁺, CD8⁺, CD4⁺CD25⁺ and CD4⁺CD25⁺Foxp3 regulatory T cells and B cells in blood and spleen. Percentage of CD3⁺ T cells in blood and spleen was not significantly different in NFATC2^{-/-} transplanted mice compared with controls (A). B cells in blood were significantly increased in NFATC2^{-/-} transplanted mice compared with controls (A). A reduction of the CD4⁺ population and an increase in the CD8⁺ population were found in blood of NFATC2^{-/-} transplanted mice (B). A significant increase in CD4⁺CD25⁺ T cells and decrease in CD4⁺CD25⁻ T cells were found in blood of NFATC2^{-/-} transplanted mice (B). Regulatory T cell population defined as CD4⁺CD25⁺Foxp3⁺ was increased in NFATC2^{-/-} transplanted mice in blood (C). Effector T helper cell population marked as CD4⁺CD25⁺Foxp3⁻ was also found to be increased in NFATC2^{-/-} transplanted mice in spleen (C). NFATC2^{-/-} chimeras did not display significant difference in percentage of CD4⁺, CD8⁺, CD4⁺CD25⁺ and CD4⁺CD25⁻ of CD3⁺ T cells in spleen (D). Values represent mean $\pm$ SEM, *P < 0.05, **P < 0.01, compared with WT.

are shown on page 173. Thus NFATC2 deficiency resulted in an enhanced migration capacity to inflamed atherosclerotic lesions, while migration to spleen was reduced.

NFAT deficiency in T-cells diminishes IL-10 production and induces T-cell proliferation without changing Treg cell function

The next question we addressed was how NFATC2^{-/-} T-cells led to more advanced lesion progression. NFATC2 deficiency did not affect Th1 (IL-6, IFN- γ and IL-12) or

Figure 5.6 Flow-cytometric analysis of the effect of leukocyte NFATC2 deficiency on naïve and memory T cell in spleen and blood. (A-C) shown are the dot plot profiles of blood cells stained with anti-CD3+ (A), anti-CD4+ and anti-CD8+ on gated CD3+ (B) and CD44 and CD62 L staining on gated CD4+ cells (C). In the CD4+ T cell population, the percentage of naïve T cells defined as CD44^{low}CD62L^{high} was reduced in blood of NFATC2^{-/-} transplanted mice compared with controls, while memory T cells defined as CD44^{high}CD62L^{low} increased (D). In CD8+ T cells, a marked decrease in naïve T cells was found in spleen. However, memory T cells in CD8+ T cells remained unchanged (E). In the CD4+CD25+ T cell population, a decrease in native T cells and an increase in memory T cells were observed in spleen and blood (F). (G-I) Flow cytometry analysis of the effect of leukocyte NFATC2 deficiency on CD62L mean fluorescence intensity (MFI) of CD62L+ T-cells in spleen and blood. CD62L MFI was significantly increased in NFATC2^{-/-} transplanted mice in CD4+CD62L^{high} (G), CD8+CD62L^{high} (H) and CD4+CD25+CD62L^{high} (I) subsets. Values represent mean $\pm$ SEM, *P < 0.05, ** p < 0.01 compared with WT.

Figure 5.7 Flow cytometry analysis of the effect of leukocyte NFATC2 deficiency on CD3 MFI of CD3+ in spleen and blood. A marked increase in CD3 MFI was observed in CD3+ T-cells in blood and spleen of NFATC2^{-/-} transplanted mice (A). (B) NFATC2 deficiency enhanced T cell mitogenic response to CD3. Assessment of the proliferation of purified CD4+CD25- T cells (³[H] thymidine incorporation) after stimulation with anti-CD3 in the presence of purified WT CD11c+ dendritic cells. An increased T cell proliferative response from NFATC2^{-/-} mice compared with NFATC2^{+/+} mice was evident. Values represent mean $\pm$ SEM, *P = 0.05, ** p < 0.01 compared with WT.

Figure 5.8 (A-D): Flow cytometry analysis of splenocytes in recipient apoE^{-/-} mice that received splenocytes labeled with CFSE isolated from either donor NFATC2^{-/-} or WT mice. Gated live splenocytes were assessed for CFSE. (A) Histogram shows splenocytes isolated from apoE^{-/-} mice without receiving CFSE+ splenocytes. (B) Representative histogram shows splenocytes of recipient apoE^{-/-} mice that receive WT CFSE+ splenocytes. (C) Representative histogram show splenocytes of recipient apoE^{-/-} mice that received NFATC2^{-/-} CFSE+ splenocytes. (D) percentage of CFSE+ cells significantly reduced in apoE^{-/-} mice that received NFATC2^{-/-} CFSE+ splenocytes. (E) NFATC2 deficiency led to enhanced migration of CFSE+ cells to atherosclerotic aortic arch lesions. CFSE+ positive cells in sections from frozen aortic were quantified. Nuclei were counterstained using Mounting Medium with DAPI. Overlay images were acquired using fluorescence microscope. The number of CFSE+ cells in atherosclerotic plaque relative to total number of cells in the plaque was significantly increased in apoE^{-/-} mice that received NFATC2^{-/-} CFSE+ splenocytes compared with apoE^{-/-} mice that received splenocytes from control group (E). F show representative overlays of images of the aortic arch from apoE^{-/-} mice that receive WT and NFATC2^{-/-} CFSE+ splenocytes, respectively. Full color figures are shown on page 173. Values represent mean $\pm$ SEM, *P < 0.05, ***P < 0.001 compared with WT.

Figure 5.9 Influence of leukocyte NFATC2 deficiency on serum cytokine levels. There were no differences in the levels of IL-6, IL-10, IFN- γ and IL-12 in serum of NFATC2^{-/-} transplanted mice compared with controls. Values represent mean $\pm$ SEM.

Th2 cytokine levels (IL-10) in circulation (Figure 5.9). Crude splenocytes isolated from NFATC2^{-/-} and WT mice were then studied for PMA and ionomycin stimulated-cytokine secretion. Although NFATC2 deficiency did not alter IFN- γ secretion ($n=9$ mice), it significantly reduced IL-10 secretion (822 ± 81 pg/ml for control vs 349 ± 47 pg/ml for NFATC2^{-/-}, $p=0.001$, Figure 5.10A). Although we previously showed a significant enhanced mitogenic response of CD4⁺CD25⁻ T cells upon anti-CD3 antibody treatment, we did not observe any differences in suppressive capacity of Treg cells between NFATC2^{-/-} and WT bone marrow transplanted mice (Figure 5.10B), despite the marked increase in CD4⁺CD25⁺Foxp3 Treg numbers in NFATC2^{-/-} spleen and blood. This suggests that the increased mitogenic activity probably reflects an augmented intrinsic response of NFATC2^{-/-} T-cells to CD3 activation.

Hematopoietic deletion of NFATC2 did not alter gene expression of regulatory T cell, Th1, Th2 and migratory T cell markers in spleens

The enhanced T-cell content of NFATC2^{-/-} chimera lesions pointed to effects of NFATC2 deficiency on T-cell differentiation, migration or activation status. Therefore we studied effects of hematopoietic NFATC2 deficiency on the T-cell gene expression pattern. Expression of FoxP3, a critical transcription factor in the commitment of T lymphocytes to the Treg cell lineage^{19, 20} was not altered in NFATC2^{-/-} and WT-transplanted mice spleen. No differences were observed in CXCR3 (Th1 cell marker), CCR3 (Th2 cell marker), CCR4 (Treg marker), CCR5 (homing marker for inflammatory sites), and CCR7 (migratory T cell marker for lymph nodes) gene expression between the two groups as well (Figure 5.11).

Discussion

A critical regulator of immune responses, NFAT contributes to a variety of inflammatory and autoimmune disorders. The recent finding that NFAT activation is indispensable

Figure 5.10 Effects of NFATC2 deficiency on cytokine secretion from splenocytes (A). Isolated splenocytes were stimulated with PMA(1ng/ml) and ionomycin (500nM). Cytokine levels in the cell culture supernatants were determined by ELISA. NFATC2^{-/-} did not alter IFN- γ secretion, however it reduced IL-10 secretion compared with WT. NFATC2 deficiency reduced IL-10 production, without affecting regulatory T cell function (B). Inhibition of proliferation of CD4⁺CD25⁻ effector T cells after coculture with CD4⁺CD25⁺ Treg cells. There was no difference in regulatory T cell function in suppressing effector T cell proliferation. **P < 0.01 (Values represent the mean $\pm$ SEM), compared with WT.

Figure 5.11 CXCR3, CCR3, CCR4, CCR5, CCR7 and FoxP3, mRNA expression in spleens of mice measured by Q-PCR were compared in NFATC2^{-/-} transplanted mice and controls (normalization by the average of cyclophilin and 18S). There was no difference in gene expression levels between the two groups. Values represent mean $\pm$ SEM.

for regulatory T-cell function¹⁵ and Th2 biased immune response^{2,9} suggests a role of this transcription factor in Th1 dominated process of atherosclerosis as well²¹. Indeed we previously showed that inhibition of calcineurin/NFAT signaling by low dose FK506 inhibited the initiation and progression of collar-induced atherosclerosis¹³, although the actual molecular NFAT target mediating this effect remains to be identified. In this study, we investigated the role of a major component in NFAT signaling in T-cells, NFATC2, in atherosclerosis. Contrary to our expectations, NFATC2 deficiency in hematopoietic-lineage cells resulted in more advanced lesion development and a

dramatic increase in lesional CD3⁺ T cell content. NFATC2^{-/-} bone marrow recipients showed a higher blood B cell content than WT controls. In addition, NFATC2^{-/-} T-cells in blood had altered CD4/CD8 ratio and were considerably enriched in CD4⁺CD25⁺ and in particular in CD4⁺CD25⁺Foxp3⁺ regulatory T cells subsets, although the T-cell suppressive activity of the latter subset was unchanged.

Importantly, NFATC2 deficiency resulted in enhanced memory T cells numbers characterized by CD44^{high}CD62^{low} and reduced levels of the CD44^{low}CD62L^{high} naïve T cell phenotype in CD4⁺ and CD8⁺ T cells. CD44 expression is important for memory T cell extravasation at inflammatory sites²², whereas CD62L is responsible for homing of naïve T cells to and retention in lymph nodes²³. Central memory T cells, unlike naïve T cells, are able to respond rapidly to antigen by secreting large amount of effector cytokines such as IL-4, IL-5, and IFN- γ , and will subsequently migrate to inflammatory sites, whereas naïve T cells mainly traffic to lymph nodes^{24,25}. Conceivably, the increased CD3⁺ T cell content in inflammatory atherosclerotic lesions in NFATC2^{-/-} transplanted mice may be explained by the shifted balance between central memory and naïve T cells. This is congruent with the finding that central memory T cells are the predominant subset in chronically inflamed tissue²⁶.

The increase in the central memory T cell marker CD44 was particularly pronounced within the CD4⁺CD25⁺ T cell population. CD4⁺CD25⁺ T cells are generally regarded as regulatory T cell in nonstimulated naïve mice²⁷, but not necessarily in high-fat fed LDLr^{-/-} recipients. CD44^{hi} CD4⁺CD25⁺ T cell subset encompasses both self-antigen specific Foxp3⁺ memory Treg and Foxp3⁻ effector T cells. The former cells were recently reported to constitute a first line of tolerogenic control to self antigens²⁸. Therefore, NFATC2^{-/-} transplanted mice displayed a skewing to FoxP3⁻ memory subsets and this shift was paralleled by a concomitant increase in memory Treg, possibly representing a compensatory response to control T-cell activity.

NFATC1 and C2 are both expressed and operational in memory CD4⁺ T cells where they are, amongst others, required for IL-2 production. Enhanced NFATC1/C2 transcriptional activity was considered instrumental in the rapid response of memory CD4⁺ T cells by mediating cytokine secretion and migration²⁹. Of interest peripheral T cells from NFATC2 and C3 double knockout mice were reported to display memory phenotype as indicated by elevated levels of CD44 expression on splenocytes³⁰. This was probably in part due to the fact that NFATC2 and C3 double deficiency impairs the responsiveness of the effector T cells to Tregs³¹. Our study adds to this finding, identifying NFATC2 as key suppressor of CD44^{high}CD62L^{low} memory T cell differentiation.

Consistent with the enhanced migratory capacity of T-cells and the higher abundance of plaque T-cells in NFATC2 deficiency, our in vivo trafficking study clearly demonstrated that NFATC2^{-/-} splenocytes preferentially home to sites of inflammation such as the

atherosclerotic lesion compared with WT splenocytes. In contrast spleen grafting was strongly reduced, possibly as a consequence of enhanced accumulation of CSFE+ cells to plaques or retention in lymph nodes.

NFATC2 has been shown to directly interact with FoxP3 as part of the transcriptional program required for Foxp3 promoter *trans* activation after triggering of the T cell receptor³² and Treg function¹⁵. The balance between initiation of an inflammatory T(eff) cell versus Treg response depends on the nuclear concentration of NFAT to interact with Foxp3 or AP1³³. Pharmacological inhibition of NFAT dephosphorylation with cyclosporine A in CD4+CD25- T cell favored Treg activity¹⁴. In this study, however, we found that while CD4+CD25+ Treg cell numbers were increased in NFATC2-/-, their intrinsic suppressive activity was unaltered. Thus the stronger proliferative response of NFATC2-/- responder T cells to CD3 activation was not attributable to compromised Treg function with NFATC2 deficiency but might be associated with the enhanced CD3+ expression on T lymphocytes in these mice. As NFATC2 deficient Treg function remained intact, Treg function was not exclusively dependent on NFATC2 transcriptional activity.

It has been described that NFATC2-/- mice exhibit a Th2 dominated pro-allergic phenotype^{2,7,9}. However, NFATC2-/- transplanted mice did not show any differences in Th1 (IL-6, IFN- γ and IL-12) or Th2 cytokine levels (IL-10) in serum compared with WT controls. IL-10 secretion by PMA/ionomycin elicited NFATC2-/- T-cells was substantially lower than that by WT T-cells, pointing to a pro-atherogenic Th1 polarized T-cell response. The reduction of IL-10 secretion in NFATC2-/- splenocytes might not be caused by changes in IL-10-producing memory T cell numbers as IL-10 producing T help cells lack a memory for IL-10 expression³⁴. The effects of NFATC2 on Th2 cytokine transcription are complex and poorly understood. Anti-CD3-injected NFATC2-/- mice exhibited a striking defect in the induction of IL-4 in splenic RNA one hour after treatment. Despite an initial defect in IL-4 production, an increase in IL-4 was observed in supernatants from NFATC2-/- splenocytes 7 days after anti-CD3 stimulation⁷. As we did not include later time points to study cytokine secretion in splenocytes, we are not able to draw firm conclusions concerning the possible Th1/Th2 bias in NFATC2-/- mice.

To conclude, although NFAT family members are critical activators of immune response³⁵⁻³⁷ and NFATC2 deficiency in mice results in a Th2 biased T-cell response, leukocyte deficiency in NFATC2 deteriorated rather than ameliorated atherosclerosis and led to an accumulation of T-cells in the plaque. NFATC2 deficient T-cells displayed an augmented mitogenic response to CD3 and a memory/activated T cell phenotype,

had robust migratory capacity to atherosclerotic lesions and produced less IL-10 upon activation. The resultant proinflammatory status of NFATC2^{-/-} T-cells could well have contributed to the surprising aggravated atherogenic response observed in NFATC2 deficiency.

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6

Cathepsins In Atherosclerosis

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1. Introduction

Cathepsins are a family of proteolytic enzymes that degrade the extracellular matrix (ECM). The ECM consists of elastins, collagens, and proteoglycans. It gives anchorage, support, and structure to tissue and functions as an adhesive substrate for vascular endothelial cells (EC) and smooth muscle cells. ECM remodeling is critically involved in many physiological and pathological processes such as wound healing, tumor growth¹, chronic inflammatory diseases such as rheumatoid arthritis², neurological disorders^{3,4} and also in cardiovascular pathologies such as atherosclerosis. Degradation of ECM in the vessel wall enables smooth muscle cells (SMC) to migrate from the media into the intima and inflammatory cells to infiltrate from the circulation into the arterial wall, processes critical in the pathogenesis of atherosclerosis. Moreover, within the plaque, ECM degradation causes thinning of the fibrous cap, often resulting in plaque rupture and thrombosis. When an occlusive thrombus forms, clinical complications of atherosclerosis with a high morbidity and mortality, such as myocardial infarction and stroke occur.

The term “cathepsin” represents lysosomal proteolytic enzymes irrespective of their enzyme category. Based on their catalytic actions, cathepsins are classified into cysteine (cathepsins (cat)B, C, F, H, K, L, O, S, V, X, W), serine (catA and G) and aspartate (catD and E) proteases^{3, 5}.

The function of cathepsins and their most well known inhibitor cystatin, in ECM degradation, vascular remodeling and atherosclerosis has been demonstrated in human and animal models. This chapter will emphasize and discuss the importance of cathepsins in atherosclerosis.

2. Synthesis and activity

Under physiologic conditions, cathepsins are localized intralysosomally. Although cathepsins were originally shown to be active in lysosomes and endosomes and execute unspecific proteolysis⁶, there is growing evidence that cathepsins can function outside lysosomes or endosomes⁷. In response to certain signals such as inflammatory cytokines interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ)⁸, angiotensin II⁹, or oxidized low density lipoprotein (oxLDL)¹⁰, they are released from the lysosomes into the cytoplasm where they activate various biological and pathological pathways including ECM degradation, inflammation and apoptosis. For example, disruption of lysosomes results in translocation of cathepsins to the cytosol and induction of apoptosis through a caspase-dependent mechanism¹¹. Cathepsins can also exert specific functions in the nucleus, and even in the mitochondrion^{12, 13}.

3. Regulation

Control and regulation of proteolytic activity are indispensable processes. Failure of these processes inevitably result in various fatal pathologies such as metastasis of cancer cells or inflammation¹⁴. Cathepsins are regulated by endogenous cathepsin inhibitors called cystatins. In general, cystatins function as a protection against the irregular release of peptidases such as cathepsins from the lysosome during apoptosis or phagocyte degranulation¹⁵. They also serve as defense against proteases secreted by proliferating cancer cells or by invading organisms, such as parasites¹⁶.

The cystatin family is divided into four sub-families^{16, 17}: the stefins (type 1), the cystatins (type 2), the kininogens (type 3) and various structurally related but noninhibitory proteins (type 4). Type 1 cystatins are cystatins A (stefin A) and B (stefin B), which are present mainly intracellularly, but can also appear in body fluids at significant concentrations. Type 2 cystatins, including cystatin C, are found in most body fluids, and mainly operate extracellularly. Type 3 cystatins include kininogens, which are present in blood proteins. Among these cathepsin inhibitors, cystatin C is the most potent inhibitor^{16, 18}, with the greatest inhibitory properties to cathepsins L and S, followed by cathepsins B and H¹⁵.

4. Cathepsins in (patho)physiological tissue remodeling

Cathepsins not only function in intra-lysosomal protein degradation, but also contribute to tissue remodeling by degrading the ECM. As cathepsins are expressed in several cell types, they participate in various tissue remodeling processes (Table 6.1). CatK is the most abundant cysteine protease expressed in osteoclasts and is instrumental in bone matrix degradation necessary for bone resorption¹⁹. CatS is expressed and secreted by the human adipose tissue and is up-regulated in obesity²⁰. The increase of catS in adipose tissue causes local degradation of fibronectin network, a key preadipocyte-ECM component, supporting the development of fat mass²¹. In addition, circulating levels of catS were also increased in obese subjects²⁰. CatS mRNA or protein in the left ventricular tissues was more abundant in rats or humans with heart failure compared to control, suggesting that catS participates in pathological left ventricular remodeling²². Patients with heart failure were found to have increased expression of catB in the myocardium, suggesting that catB plays a role in the development of heart failure²³. Furthermore, enhanced expression of several cathepsins (B, D, L and S) was observed in several types of tumors, and are thought to contribute to tumor growth and metastasis.^{24, 25} Increased catD expression was present in activated astrocytes in neurodegenerative diseases, such

as scrapie in mice and Alzheimer’s disease in human. The increase in cathepsin D may be an ongoing response to the deposition of abnormal aggregated proteins that have neurotoxic effects²⁶.

Some *in vitro* and *in vivo* studies suggested that cysteine proteinases play an important role in renal pathophysiology. CatB, H, and L were present in glomeruli and other fractions prepared from normal rat kidney and were able to degrade the intact glomerular basement membrane *in vitro*²⁷.

Table 6.1 Cathepsins in tissue remodeling

Tissue remodeling	Description
Bone resorption	catK
Fat tissue turnover	catS
Cardiac remodeling	catB and S
Tumor	catB, D, L, and S
Neurodegeneration	catD
Renal pathology	catB, H and L

5. Cathepsins in atherosclerosis

5.1 Expression of cathepsins in human atherosclerosis

Considering the widespread functions of cathepsins in the different pathologies, it is not surprising that cathepsins play a key role in cardiovascular disease. In atherosclerotic arteries, cathepsins are expressed by most of the plaque cell types, including macrophages, ECs and SMCs^{28, 29}. Various studies showed that cathepsin B³⁰, F³¹, L³², K³³ and S⁸ mRNA or protein level were increased in either human or mouse atherosclerotic lesions, whereas they were only weakly expressed in normal arteries. Cathepsins F, L, K and S were mainly found in macrophages, SMCs and ECs. Specifically, macrophages in the shoulder region of human atheroma contained abundant catK and S⁸. Likewise, SMCs that appeared to transverse the internal elastic laminae and of the fibrous cap also expressed catK and S⁸, suggesting that SMC and macrophages utilize these cathepsins to enter the atherosclerotic plaque. ECs aligning the lumen of the vessel itself and the intraplaque microvessels in human atheroma lesions expressed catS²⁹, suggesting an involvement of this protein in neovascularization.

The enhanced cathepsin expression in human and murine atherosclerotic lesions suggests an involvement of cathepsins in the process of atherosclerosis. The next

paragraphs outline the consequences of cathepsin deficiency on plaque progression and plaque phenotype (5.2) and describe the potential mechanisms how the different cathepsins affect atherogenesis (5.3; Figure 6.1).

5.2 *The role of cathepsins in atherogenesis: lessons learned from animal models*

Cathepsin S deficiency in LDL receptor (LDLR^{-/-}) mice protected against atherosclerosis: reducing atherosclerotic plaque area, plaque progression, the number of elastin breaks and elastase activity. Furthermore, catS deficiency induced a reduction in SMC and collagen content and decreased fibrous cap thickness³⁴. CatS deficiency in apolipoprotein E deficient (apoE^{-/-}) mice was also found to reduce plaque ruptures, defined as visible defects in the cap of atheromatous lesions and accompanied by intrusion of erythrocytes into the region below⁸. In addition, catS expression in macrophages colocalized with areas of elastin fragmentation in mice³⁵.

CatK deficiency was found to protect against ECM remodeling in atherosclerosis as well. CatK deficiency in apoE^{-/-} mice resulted in a 42% reduction in atherosclerotic plaque area; although the total number of plaques remained unchanged, there was a relative increase in early lesions and a relative decrease of the number of advanced lesions when catK was absent³³. Furthermore, catK deficiency led to an increase in collagen content and macrophage size, and a decrease in elastin breaks³³. These results were confirmed by Samokhin *et al* who demonstrated that catK deficiency inhibited plaque progression and increased fibrous cap thickness in the brachiocephalic artery after high fat feeding³⁶. Interestingly, opposite results were found when only leukocyte-catK was absent. In a bone marrow transplantation model in which catK deficient bone marrow was transplanted into irradiated atherosclerotic LDLR^{-/-} mice, atherosclerotic lesions had a vulnerable atherosclerotic plaque phenotype with reduced collagen levels, an increase in macrophage content and an accelerated necrotic core formation³⁷. However, these data are supported by the paper of Lutgens *et al*, where catK deficient macrophages showed an increase in lipid uptake and foam cell formation^[33].

Atherosclerotic lesions in either human or apoE^{-/-} mice have comparatively low levels of cystatin C (cathepsin inhibitor), whereas normal arteries have abundant cystatin C expression in medial SMCs and in ECs^{29, 38}. Deficiency of cystatin C in apoE^{-/-} mice significantly increased plaque size³⁹ and increased elastolytic activity⁴⁰, suggesting an important role played by cystatin C in atherosclerosis development.

All the above findings demonstrate the importance of cysteine proteases and the cathepsin inhibitor cystatin C in atherosclerosis.

5.3 *Cathepsins in atherosclerosis: potential mechanisms*

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5.3.1 Cathepsins are regulated by shear stress

The mechanic force (shear stress) that is constantly present over vascular ECs regulates the structure and function of ECs⁴¹. This mechanic force is generated by blood flow over the vascular endothelium and tends to develop different patterns of hemodynamic force based on the region and curvature of the artery. Oscillatory shear stress, which occurs in branched or curved regions, is associated with pro-atherogenic events. Laminar shear stress occurs in “straight” arteries, and is associated with low susceptibility to atherosclerosis⁴¹. Platt *et al* showed that atheroprotective laminar shear stress decreased catL activity, while pro-atherogenic oscillatory shear stress significantly enhanced catL activity compared with laminar shear stress. These observations suggested that cathepsin L could participate in the development of vascular remodeling and atherosclerosis partly by a shear stress sensitive response⁴². CatK was also found to be mechanosensitive. Laminar shear stress decreased catK protein expression in ECs *in vitro* compared to oscillatory shear stress, suggesting a potential role for catK at sites of disturbed flow associated with vascular pathology. This study also showed a positive correlation between catK levels in endothelium and human atherosclerotic lesion development, supporting the evidence for a role of catK in atherosclerosis formation. The above findings suggest that catL and K expression in ECs are regulated by shear stress and partially contribute to the shear-dependent regulation of the ECM protease activity leading to vascular remodeling and atherosclerosis.

5.3.2 Cathepsins mediate leukocyte and SMC Migration

One of the first signs of atherosclerosis is recruitment of leukocytes from the vascular lumen into the arterial wall, followed by adhesion and migration through the endothelial layer and arterial basement membrane⁴³. Blood-born monocytes use cathepsins to degrade ECM and migrate through endothelial layers. For example, catS-deficient monocytes displayed less migration through a SMC layer than wild-type monocytes *in vitro*³⁴.

The role of cathepsins in leukocyte migration has also been illustrated in Jurkat T cells⁴⁴. The migration activity of T lymphocytes through ECM did not require ECM degradation but was mediated by adhesion molecules and cytoskeletal rearrangements. CatX overexpressing T lymphocytes displayed polarized migration-associated morphology and enhanced migration on 2D and 3D models using intercellular adhesion molecule 1 (ICAM-1) and Matrigel-coated surfaces. The proteolytic activity of catX was not involved in the increased invasiveness of catX overexpressing cells. Instead, an active form of catX colocalised with lymphocyte-function-associated antigen 1 (LFA-1) in migrating cells. LFA-1 is found on leukocytes and binds to ICAM-1 on antigen-presenting cells

and functions as an adhesion molecule⁴⁵.

In addition to the migration of monocytes into the arterial wall, migration of SMCs from the media into the intima also play a key role in atherosclerotic lesion formation, as well as in restenosis^{46, 47}. Migration of SMCs from the medial to the subendothelial space requires degradation of ECM. The interaction of catS with ECM components during SMC migration was examined by Cheng *et al.* A selective cat S inhibitor- (Morpholinurea-leucine-homophenylalanine-vinylsulfonephenyl) and the endogenous inhibitor cystatin C significantly attenuated SMC invasion through ECM, suggesting that cathepsins released from SMCs contribute to SMC invasion through collagen and elastin substrates. Western blot analysis on subcellular fractions showed that the active form of catS was present on the SMC plasma membrane but not in the cytosol. In contrast, the active forms of catK, B, L, and D were not expressed on the SMC plasma membranes. These findings suggested that membrane-bound catS facilitated SMC proteolytic activity and thereby SMC invasion through ECM. This notion was further confirmed by the finding of partial co-localization of catS and integrin $\alpha\beta 3$ at the cell surfaces. $\alpha\beta 3$ integrin is a receptor for several proteases such as matrix metalloproteinases (MMPs)⁴⁸⁻⁵⁰ and involved in SMC adhesion and migration. Thus catS might cooperate with integrin $\alpha\beta 3$ to facilitate SMC invasion. In the study by Cheng *et al.*, the inhibition of catS reduced SMC invasion through ECM but had no effect on adhesion or on migration through ECM. It suggests that catS likely contributes to the proteolytic process during SMC invasion through ECM but does not play a role in mediating the migration process itself⁵¹.

5.3.3 Cathepsin-induced ECM degradation in atherosclerosis

ECM degradation plays an important role in development and destabilization of the atherosclerotic plaque⁵². Modifications in ECM homeostasis, as a consequence of alteration in the degradation and/or synthesis of the vessel wall ECM, have been associated with vascular diseases. Plaque formation occurs as a result of SMC migration and proliferation accompanied by degradation of ECM. In the latter stages of atherosclerosis, thrombotic complications frequently develop from rupture of the fibrous cap or superficial erosion of the endothelium, both of which are the result of ECM degradation.

Cathepsins were found to exert strong elastinolytic and collagenolytic activity in culture media conditioned by various cell types. Cultured SMC stimulated with cytokines, secreted active catB and L and degraded substantial amounts of insoluble elastin^{8, 32}. Monocyte-derived macrophages synthesized not only metalloproteinases, but also cat

B, L, and S. However only the cathepsins were detected in the extracellular milieu, and macrophage-mediated elastolytic activity was completely abrogated by inhibiting catL and S but not by MMP inhibitors⁴⁴. Cultured EC stimulated with pro-inflammatory cytokines or growth factors induced the expression of catL, and enabled catL dependent degradation of extracellular collagen and elastin⁵³. These findings suggested that SMC, macrophages and EC use cysteine proteinases-dependent processes for ECM remodeling and this contributes to atherosclerosis formation.

Besides the above *in vitro* data, one *in vivo* study also suggested that cathepsins play important roles in ECM degradation. In addition to abundant expression of catK and S in macrophages and intima SMCs, extracts from human atheroma showed a two-fold increase in elastolytic activity compared with normal vessels, which could be inhibited significantly by a cysteine protease inhibitor E64⁸.

5.3.4 Cathepsin mediated neovascularization

The microvascular network of vasa vasorum is found in the adventitia and the outer media of normal vessels. However, during atherosclerotic plaque development, these networks grow and expand into the plaque⁵⁴. Plaque neovascularization is thought to contribute to lesion progression in various ways. It offers a port of entry for leukocyte and plasma constituents such as lipoproteins into atherosclerotic lesions^{55, 56}. Furthermore, the fragile nature of these neovessels can lead to focal intraplaque hemorrhage, which further promotes inflammation and thrombotic complications of atherosclerosis⁵⁷. The effects of cathepsins on neovascularization have been studied intensively in tumors and wound healing, but less in cardiovascular diseases. Cathepsin activity has been shown to be critical during tumor invasion and angiogenesis⁵⁸, which requires lysis of the ECM to pave the way for neovasculature⁵⁹ and proteolysis of the endothelial basement membrane⁶⁰.

The role of cathepsins has been further investigated in the context of endothelial progenitor cells (EPC)-mediated neovascularization. EPCs have been shown to improve neovascularization in ischemic tissues^{61, 62}. Gene expression profiling of EPCs and EC showed that catL was highly expressed in EPCs compared to EC. Mice receiving cat L deficient bone marrow cells showed impaired functional recovery following hind limb ischemia. However, inhibition of other proteases such as catS and D, and MMPs did not affect EPC metalloproteinase activity⁶³, suggesting that cathepsin L is specifically required for EPC-mediated neovascularization. Endothelium-derived cathepsins may also contribute to angiogenesis as well. CatS inhibition reduced microtubule formation in ECs by impairing cell invasion. Furthermore, catS deficient mice displayed fewer

microvessels in healing wounds⁶⁴. Taken together, these data suggested that cathepsins are involved in the processes of neovascularization. However, until now, direct evidence for a significant role for cathepsins in plaque neovascularization is lacking.

5.3.5 *Cathepsins mediate inflammation*

Atherosclerosis is an inflammatory disease in which both innate and adaptive immune responses are involved⁶⁵. Innate, but also adaptive immune responses such as antigen presentation occur within the atherosclerotic plaque, which is rich in T lymphocytes, but also in antigen-presenting macrophages and dendritic cells⁶⁶. In addition, to proteolytic activity, cathepsins also play roles in inflammatory processes involving both innate immunity, such as Toll-like receptor-9 (TLR9) signaling and processes of adaptive immunity, including antigen presentation.

5.3.5.1 *Cathepsins in innate immunity: TLR9 signaling*

Matsumot *et al* found that catB and L inhibitors suppress the interaction of CpG-B (TLR ligand) with TLR9 in 293T cells, suggesting a role for cathepsins in regulating CpG-B-TLR9 interaction, and thus innate immunity⁶⁷. Moreover, catS and F, but not H, are able to complement TLR9 responses in Ba/F3 cells, which are defective in TLR9 responses (unable to activate NF- κ B in response to CpG-B)⁶⁷.

The involvement of cathepsins in TLR9 signaling was also present in dendritic cells⁶⁸. Administration of a potent orally active catK inhibitor named NC-2300 in rats with adjuvant-induced arthritis not only suppressed bone resorption by osteoclasts, but also showed anti-inflammatory effects, resulting in reduced paw swelling. Further studies showed that catK inactivation led to the blockade of essentially all the downstream pathways of TLR9 signalling in dendritic cells, showing a crucial role for cathepsin K in TLR9 signaling.

TLRs, especially TLR1, 2, 4, have been recognized for their roles in atherosclerotic lesion development and progression⁶⁹. Unfortunately, the involvement of TLR9 signaling in atherosclerosis is not yet clear. Further research on the role of cathepsin-mediated TLR9 response in the development of atherosclerosis is warranted.

5.3.5.2 *Cathepsins and antigen presentation*

The function of cathepsins in antigen presentation has been intensively studied. MHC class II molecules present antigen peptides on the surface of antigen presenting cells (APC), which are recognized by CD4⁺ T cells. The cooperation of invariant (Ii) chain with MHC class II dimers is required for proper antigen presentation to CD4⁺ T cells. The Ii cytoplasmic tail targets the MHC classII-Ii complex to the endosomal pathway

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and prevents early loading of antigenic protein on MHC class II with class II-associated invariant chain peptide. Degradation of Ii is an important regulatory step in the maturation of MHC class II dimers. Maturation of the endosome leads to the activation of lysosomal enzymes, which degrade Ii from class II-Ii complexes and induce subsequent maturation of class II molecules. CatF, L, S, K, and V have all degrade MHC II-associated Ii in professional APC and are thus required for antigen processing⁷⁰.

Compared with other lysosomal cysteine proteases, catS has displayed some unique characteristics in that it can not only degrade MHC II-associated Ii in professional APC, but also in non-professional APC such as intestinal epithelial cells^{71, 72}. Several studies describe the functional significance of cathepsin inhibition in antigen presentation, thus inhibition of catS reduced autoantigen presentation and development of organ-specific autoimmunity in mice^{73, 74}.

In atherosclerosis, a well known auto-antigen in the cellular immune response of atherosclerosis is oxLDL⁷⁵. Anti-oxLDL antibodies were detected within rabbit and human atherosclerotic lesions⁷⁶. In addition, anti-oxLDL antibodies titers significantly correlated with the extent of atherosclerosis in mice⁷⁷. Moreover, immunization with splenic B cells led to the production of anti-oxLDL antibodies, which conferred protection against atherosclerosis in mice⁷⁸. Interestingly, cathepsin S deficient LDLr^{-/-} mice showed significant reduction in atherosclerotic lesion size and lower titers of autoantibody against both malondialdehyde-oxLDL and copper-oxLDL epitopes²⁹.

5.3.5.3 Cathepsins mediate TGF- β signaling

Cathepsins might be involved in anti-inflammatory responses via the TGF- β pathway. Lutgens *et al* showed that besides reduced atherosclerotic lesion size, deficiency of catK induced expression of genes involved TGF- β signaling in atherosclerotic lesions⁷⁹. As inhibition of TGF- β in apoE^{-/-} mice initiated an inflammatory plaque phenotype⁸⁰, induction of TGF- β signaling might partly explain the protective effect of catK deficiency in atherosclerosis formation.

5.3.6 Apoptosis

Apoptosis of foam cells contributes to necrotic core formation, a hallmark of plaque severity. Several cysteine proteases have been described to participate in this process⁸¹⁻⁸³. Z-FAFMK (a selective inhibitor of catB and L) prevents oxysterol-induced apoptosis of mononuclear cells⁸⁴. The expression of catL in human atherosclerotic plaques was correlated with apoptosis, suggesting that catL is involved in macrophage apoptosis. Moreover, macrophage apoptosis in atherosclerotic coronary artery specimens was significantly correlated with expression of catL in cell membranes and nuclei⁸⁵.

Furthermore, selective catS inhibition by 05141 and a null mutation of catS, ameliorated IFN- γ -induced apoptosis as manifested by reduced activation of caspases-3, -8, and -9 and reduced expression of Bim, Bid, Fas, Fas ligand, TNF- α , TNF-related apoptosis-inducing ligand, and protein kinase C- δ in lung. These observations highlight the suppressive effects of catS inhibitor in IFN- γ -induced apoptosis activation via both the intrinsic/mitochondrial and extrinsic pathways⁸⁶.

The mechanism by which cysteine proteases induce apoptosis is thought to involve both caspase-dependent and caspase-independent cell death pathways⁸⁷. Cathepsins are located in the lysosomes under physiological conditions. Nonetheless, oxidative stress causes lysosomal leakage and rapidly initiates cathepsin translocation from lysosomes to the cytosol and participation in both apoptotic pathways. The caspase-dependent pathway includes a direct cleavage of Bid and/or Bak/Bim. Translocation of these pro-apoptotic proteins to the mitochondrial outer membrane leads to release of apoptotic factors such as cytochrome C. This results in an indirect activation of caspases and subsequent apoptosis. Cathepsins are also able to directly cleave caspases, which is followed by cleavage of Bid and/or Bax, translocation of these proteins to mitochondria and subsequent downstream events that cause apoptosis. Cathepsins are also able to trigger the release of apoptosis-inducing factor and cause caspase-independent cell death^{84, 88, 89}.

5.3.7 Lipid metabolism

Lipid retention in macrophage derived foam cells is one of the hallmarks of atherosclerotic plaque development and progression. Cathepsins have been shown to affect foam cell formation bivalently. Some cathepsin family members enhance foam cell formation, while others impair the formation of foam cells²⁸.

5.3.7.1 Cathepsins reduce foam cell formation

Cathepsins are able to degrade (modified) LDL. CatB inhibition reduced modified LDL degradation in human aortic SMC lysates. Decreased lysosomal degradation may cause LDL accumulation in SMCs and subsequent foam cell formation⁹⁰. Several studies showed that catK deficiency was able to increase lipid uptake and consequently foam cell formation. Bone marrow-derived macrophages from catK deficient apoE^{-/-} mice showed an increased uptake of modified LDL³³. Pathway analysis revealed that the increased lipid uptake was regulated by both CD36 and caveolins⁷⁹. Furthermore, bone marrow-derived macrophages from catK deficient apoE^{-/-} mice showed an increase in cholesterol ester accumulation compared with apoE^{-/-} bone marrow-derived macrophages, which was stored in lysosomal compartments³³. These data indicate that

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catB and K are able to reduce foam cell formation by degrading (modified) LDL and inhibiting lipid uptake.

5.3.7.2 Cathepsins stimulate extracellular lipid accumulation and foam cell formation

In vitro studies showed that recombinant catF strongly degraded apoB-100³¹. Degradation of apoB-100 by catF induced accumulation and fusion of LDL particles and enhanced the ability of LDL to bind proteoglycans, consequently leading to the accumulation of extracellular lipid droplets³¹. This study indicates that cathepsins could contribute to extracellular lipid accumulation in the arterial wall, an important characteristic of atherosclerosis. Degradation of cholesterol acceptors by cathepsins not only induced extracellular lipid accumulation, but also inhibited lipid efflux. CatS completely (100%) degraded apolipoprotein A-1 (apoA-1), which caused total loss of the ability of apoA-1 to induce cholesterol efflux. Cathepsins F and K incompletely degraded apoA-1 leading to a reduction of cholesterol efflux by 30% and 15% respectively⁹¹. These data suggest that cathepsin-mediated degradation of cholesterol acceptors can inhibit lipid efflux and thereby aggravate foam cell formation.

5.3.8 Thrombus formation

Cardiovascular disease is characterized by end-stage thrombotic complications, either due to rupture of the fibrous cap or superficial erosion. CatG is known to be involved in thrombosis. After release from neutrophils, catG leads to calcium mobilization via the catG platelet receptor (protease-activated receptor 4)⁹², thereby inducing platelet aggregation⁹³. CatG can also affect the morphological integrity of EC through a mechanism involving vitronectin-bound plasminogen-activator-inhibitor-1 (PAI-1) detachment. The detachment of PAI-1 from the subendothelial matrix induces F-actin cytoskeleton rearrangement with consequent changes in morphological integrity of EC. These events expose a highly thrombogenic surface to which platelets can adhere and become activated⁹⁴.

Despite large amount of data implying a functional role of catG in thrombosis formation, catS seems to have anti-thrombotic property. CatS-deficient mice had accelerated thrombotic responses to artery injury and shorter plasma clotting time²⁹. The mechanism of catS on thrombus formation in atherosclerosis development however needs to be further investigated.

5.4 Cathepsins in plaque instability

Rupture of atherosclerotic plaques is the most common cause for thrombotic

complications, resulting in high morbidity and mortality⁹⁵. Rupture-prone lesions are characterized by a large lipid core and a thin fibrous cap. Morphological studies showed that cathepsins were particularly expressed in macrophages in the shoulder regions of plaques, an area prone to rupture³⁵. In a human study, comparison of gene expression in a stable versus a ruptured (an ulcerated surface with or without thrombosis or hemorrhage) atherosclerotic plaque obtained from the same patient, identified significantly greater amounts of catB (mRNA and protein) and catS (mRNA) in ruptured segments compared with stable segments⁹⁶. Furthermore, catL was significantly increased in atherosclerotic plaques containing a large necrotic core and a ruptured plaque, suggesting that catL was involved in the development of unstable plaques⁹⁴. It is likely that the presence of cathepsins in atherosclerotic lesions can degrade ECM and thereby contribute to plaque vulnerability. In contrast, upregulation of catK was identified in advanced stable plaques compared with plaques containing thrombus⁹⁷.

6. Therapeutic potential

Given their role in bone resorption, cathepsins have been considered valuable therapeutic targets in osteoporosis by pharmaceutical companies. Several catK inhibiting compounds in clinical trials to study their effects on osteoporosis or osteoarthritis, have inhibited bone resorption and improved bone formation^{98, 99}. The recent discovery of the involvement of cathepsins in atherosclerosis development directed attention of pharmaceutical companies to investigate the therapeutic potential of cathepsin inhibitors on the initiation and/or progression of atherosclerosis. Until now, one cathepsin K inhibitor compound was patented and claimed to be useful for treating or preventing atherosclerosis and atherosclerotic cardiovascular disease (<http://www.wipo.int/pctdb/en/wo.jsp?WO=2006076797>). However, before catK inhibitors are suitable for treating patients with cardiovascular disease, mechanisms on its detrimental effects on foam cell formation should be further investigated.

7. Conclusions

In the past years, several studies have indicated important roles for cathepsins in the process of atherosclerosis. Their capacity to degrade ECM paves the way for monocyte recruitment from the circulation into the intima and for SMCs to migrate from the media into the intima. In addition, cathepsins cooperate with adhesion molecules such as LFA1 and $\alpha v \beta 3$ during leukocyte invasion and modulate cytoskeletal rearrangement. Within the atherosclerotic lesion itself, ECM degradation induces rupture of the fibrous cap. Cathepsins are also implicated in several inflammatory

responses. Cathepsins are needed for processes involved in both innate immune reactions such as TLR3, 7, and 9 responses and adaptive responses by participating in antigen presentation, processes crucial in atherosclerosis. Furthermore, cathepsins might affect sprouting of neovessels of the existing vasa vasorum into the plaque, apoptosis, foam cell formation and thrombus formation and thereby contribute to atherosclerotic plaque vulnerability.

As shown in this review, the different members of the cathepsin family are very versatile molecules, modulating proteolysis, inflammation, lipid uptake by macrophages, apoptosis and coagulation, all processes known to work in concert during the process of atherosclerosis. Therefore, cathepsin-antagonists are considered an attractive treatment for atherosclerosis. Several of these antagonists have been patented and further patient-related studies need to be performed to reveal the value of cathepsin-antagonists in preventing morbidity and mortality as a result of clinical complications of atherosclerosis.

Figure 6.1 Overview of potential roles of cathepsins in atherosclerosis formation. Overview of potential roles of cathepsins in atherosclerosis formation. Cathepsins are expressed in ECs, SMCs, macrophages and foam cells. Cathepsins are able to degrade ECM containing elastin and collagen, which facilitate SMC to migrate from media and monocyte and T lymphocytes from circulation to intima. ECM degradation by cathepsins might facilitate neovessel formation. Both macrophages and SMCs take

up oxLDL and become foam cells, these processes involve cathepsins as well. The apoptosis of foam cells contribute to necrotic core formation, cathepsins might participate in this process. Cathepsins might compromise anti-oxLDL antibody production by interfering with antigen presentation process. Cathepsin may also induce plaque rupture and thrombosis formation.

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7

Cathepsin K gene disruption does not affect murine aneurysm formation

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Abstract

Cathepsin K (catK), a lysosomal cysteine protease, exerts strong elastinolytic and collagenolytic activity and is implicated in a range of pathological disorders including cardiovascular disease. CatK expression was found to be elevated in human aortic aneurysm pointing to a role in this vascular disease. In the angiotensin II (Ang II)-induced mouse model for aneurysm formation, catK, S and C expression was strongly upregulated. Therefore, we investigated the effect of catK deficiency on Ang II-induced aneurysm formation in the abdominal aorta of apoE^{-/-} mice.

Contrary to our expectations, catK deficiency did not protect against aneurysm formation, nor did it affect medial elastin breaks. Proteolytic activity in abdominal aortic lysates were comparable between apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice. Adventitial presence of catS and catC-expressing cells was significantly increased in catK^{-/-}/apoE^{-/-} versus apoE^{-/-} mice, which might have compensated for the deficiency of catK-derived proteolysis in the aneurysm tissue of catK deficient apoE^{-/-} mice. Circulating granulocytes and activated T cell numbers were significantly increased in Ang II-infused catK^{-/-}/apoE^{-/-} mice, which was consistent with the borderline significant increase in adventitial leukocyte content in catK^{-/-}/apoE^{-/-} compared to apoE^{-/-} mice. Strikingly, despite unchanged proteolytic activity in abdominal aortic aneurysms (AAA) lesions, collagen content in the aneurysm was significantly increased in catK^{-/-}/apoE^{-/-} mice. In conclusion, while catK deficiency has major impact on various vascular pathologies, it did not affect murine aneurysm formation.

Introduction

AAA are permanent dilations of the arterial wall in the abdominal aorta¹, which after rupture can cause life-threatening bleeding. The prevalence of AAA in western society, as diagnosed by means of autopsy, ultrasound screening, and hospital discharge data, is substantial, ranging from 19-34% in women to 66-81% in men². Women are usually older when they undergo AAA repair². Although a history of atherosclerotic disease predisposes to aneurysm formation, several other factors contribute to its ontogenesis, including genetic predisposition, inflammation, hypertension and hyperlipidemia^{3,4}.

Excessive degradation of extracellular matrix (ECM) components such as elastin and collagen^{5,6}, a critical process in AAA, was shown to be at least in part dependent on a local disbalance between matrix metalloproteinases (MMPs) and cognate inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Indeed doxycycline, a broad spectrum MMP inhibitor, was shown to prevent Ang II-induced aneurysm formation in hyperlipidemic LDL receptor deficient mice⁷. In addition to MMP, other cysteine proteases like cathepsins are involved in ECM degradation⁸.

CatK is a papain-like cysteine protease that has a distinct ECM degrading potential. It is one of the most potent elastases in mammals, and in addition harbors unique collagenolytic activity^{9,10,11,12}. In AAA wall, protein levels and activities of catK, L and S were elevated compared to that in healthy arteries, whereas levels of the endogenous cysteine protease inhibitor cystatin C were decreased^{13, 14,15}. In a previous study, we already showed that catK deficiency in apoE^{-/-} mice reduced plaque progression with a concomitant increase in plaque collagen content and macrophage size¹⁶. In addition, the number of elastin breaks in the media underlying the atherosclerotic plaque was sharply decreased in advanced lesions of catK^{-/-}/apoE^{-/-} compared to apoE^{-/-} mice. Taken together, the profound effect of catK on atherosclerosis, an established risk factor for AAA, and its enhanced expression in human aneurysms point to a potentially important, adverse role for catK in aneurysm formation. This hypothesis was tested in an established mouse model of AAA in which aneurysm formation is induced by sustained subcutaneous infusion of Ang II in apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice. Surprisingly, our data showed that catK deficiency neither reduced aneurysm formation nor reduced proteolytic activity in abdominal aortic lysates, although we did notice increased collagen content in the aneurysm itself. Remarkably, catS and C staining revealed a more abundant presence of catS and catC-positive cells in the adventitia of catK deficient animals. Apparently, in the absence of catK, other cysteine proteinases may compensate for the loss in catK-derived proteolytic activity in the arterial wall. Moreover, catK deficiency dramatically increased circulating granulocyte and activated T cell numbers in Ang II-infused but not in untreated mice, which might counteract the potentially protective pro-fibrotic effect of catK deficiency in AAA.

Materials and Methods

Animals

Male apoE^{-/-} mice on a C57Bl6 background were obtained from IffaCredo (Lyon, France). The catK^{-/-} mice, kindly provided by Dr P Saftig, were generated on an outbred 129SVJ-C57Bl/6J genetic background¹⁷. After import, we subsequently intercrossed the catK^{-/-} mice at least 9 times with apoE^{-/-} mice to generate catK^{-/-}/apoE^{-/-} mice and syngenic catK^{+/+}/apoE^{-/-} control mice. Animals were maintained in accordance with the Dutch government guidelines and animal experiments were approved by the regulatory authority of the University of Maastricht. Mice were fed a normal chow diet throughout the experiment.

Induction of aneurysm formation by chronic Ang II infusion

Aneurysm formation was induced by subcutaneous Ang II infusion¹⁸. Alzet mini-osmotic pumps (Alze type 2004) were implanted into apoE^{-/-} (n=19 mice) and catK^{-/-}/apoE^{-/-} mice (n=20 mice) at 10 weeks of age. Pumps were filled with Ang II (Sigma) dissolved in phosphate buffered saline (10 mM sodium phosphate, 150 mM sodium chloride; pH=7.4) to mediate the subcutaneous delivery of 1 ng/g/min of Ang II for 28 days. Pumps were placed subcutaneously in the neck of anesthetized mice through a small incision that was closed using a silk suture (5-0).

Tissue harvesting and histological analysis

After 4 weeks of Ang II infusion, mice were sacrificed and the arterial system was perfused with PBS containing 0.1 mg/ml nitroprusside (Sigma) through a catheter inserted into the left cardiac ventricle and subsequently with 1% paraformaldehyde. The aortic piece comprising the ascending aorta to the aortic bifurcation was fixed overnight in 1% paraformaldehyde and embedded in paraffine. We also investigated aortic tissue from mice which died before sacrificing. Ten cross-sections (4 µm thick) were cut at 200 µm intervals throughout the abdominal aorta starting at the middle of the aneurysm. From each level, a cross-section was stained with Elastica-von Gieson (EvG) for total vessel area and number of elastic lamina breaks. Transmural breaks (complete medial disruption) and more focal destructions of single elastic lamina were both scored as elastin break but at a different extent. More specifically, a transverse section containing a focal destruction of a single elastic lamina was scored as one break; while a transmural break encompassing four to five elastic lamina breaks were counted as four or five breaks, respectively.

The relative collagen area in the media and the aneurysm area, i.e. the percentage of total area that stained positive for Sirius red, was determined under a microscope coupled to a computerized morphometry system (Quantimet 570, Leica). Morphometric analysis was performed by one blinded investigator (LBai; intra-observer variability was < 10%).

Quantification of aneurysm formation

Severity of aneurysms in the suprarenal region of the aorta was analyzed according to Daugherty et al¹⁹. In this scoring system, a healthy artery not obviously different from untreated apoE^{-/-} mice was classified type 0, a dilated aorta/lumen without thrombus as type I; remodeled tissue often containing thrombus as type II; a pronounced bulbous form of type II containing thrombus as type III; while lesions with multiple aneurysms containing thrombus as type IV. A numerical score was allocated to each mouse. For statistical comparison of disease progression stages, a semi quantitative pathology score was calculated by averaging the numerical aneurysm score of apoE^{-/-} and catK^{-/-} / apoE^{-/-} mice²⁰.

Immunohistochemistry

Ruptured aneurysm lesions (containing fatal and non-fatal rupture) were used to study lesion morphology. Sections were stained with the following antibodies: Mac3 rat monoclonal antibody (1:30, BD Biosciences Pharmingen) to detect macrophages, CD45 (1:5000, BD Biosciences Pharmingen) to detect leukocytes, α -smooth muscle actin (α -SMA, 1:500, Dako) to detect smooth muscle cells (SMC) and Ly-6G (1:200, BD Biosciences Pharmingen) to detect granulocytes. Immunostaining of catS and catC were performed using rabbit anti-mouse catS (1:500, Calbiochem) and goat anti-mouse catC (1:30, Santa cruz) antibodies. Mouse lymph node was used as positive control for CD45 staining, mouse spleen as positive control for Ly-6G staining, and mouse atherosclerotic lesions as positive control of both Mac3 and catS/C staining. Deletion of the primary antibody served as negative control and did not show any staining for any of the antibodies.

The section containing the largest aneurysm area was used to quantify the number of CD45⁺, Mac3⁺ and cats/C⁺ cells. The relative macrophage, leukocyte and granulocyte content in aneurysm and adventitial area was calculated by correcting the Mac3⁺ and CD45⁺ cell number for the total cell content of aneurysm and adventitia, respectively. As Ly-6G⁺ cells were not present in every slide, the number of Ly6G⁺ cells counted in 6 slides was divided by the sum of 6 corresponding aneurysm lesion area. CatC⁺ and catS⁺ cells were primarily detected in the adventitia of the aneurysm lesion. To quantify the relative catC⁺ and catS⁺ cell density, the number of catC⁺ and catS⁺ cells in either the aneurysm region or in the adventitia was divided by the total number of cells in the aneurysm and adventitia, respectively. All measurements were conducted by one investigator (LBai; intra-observer variability was < 10%).

Bone marrow-derived macrophage isolation and culturing

Bone marrow-derived macrophages were isolated from the femur and tibia of apoE^{-/-} mice. Cells were cultured in standard RPMI containing L-glutamine, HEPES, 10% fetal calf serum, 100 IU/mL penicillin/streptomycin, and 15% L929 cell conditioned medium. After 7 days of culturing, bone marrow-derived macrophages were seeded

at a density of 1×10^6 cells/ml in culture medium supplemented with 2.5 mM Ang II (Sigma) and incubated for 24h.

Quantitative RT-PCR

CatK, S and C gene expression levels were determined in bone marrow-derived macrophages exposed to Ang II (2.5 mM), in AAA tissue from Ang-II infused apoE^{-/-} mice and in abdominal aorta tissue from untreated apoE^{-/-} mice. RNA was extracted from cell or tissue lysates with Nucleospin RNA II kit (MACHEREY-NAGEL). cDNA was generated with iScript™ CDNA synthesis kit (BIO-RAD). Real-time PCR was done with a Taqman IQ™ SYBR Green Super Mix (BIO-RAD). Cyclophilin was used as a house keeping gene. Primer sequences of catK, catS, catC and cyclophilin were shown in table 7.1.

Table 7.1

	<i>Forward primer (5'-3')</i>	<i>Reverse primer (5'-3')</i>
catK	GGGCCAGGATGAAAGTTGTA	CACTGCTCTCTTCAGGGCTT
catS	AGAGAAGGGCTGCGTCACT	GATATCAGCTTCCCCGTTTTTCAG
catC	CAACTGCACCTACCCTGATC	CTCGTCGTAGGCAGTATCCA
cyclophilin	CAAATGCTGGACCAAACACAA	TTCACCTTCCCAAAGACCACAT

Collagenase and elastase activity assay

Abdominal aortas from apoE^{-/-} mice (n=7) and catK^{-/-}/apoE^{-/-} mice (n=6) were harvested four weeks after Ang II infusion and snap frozen in liquid nitrogen. Protein extracts from abdominal aortas were made using a lysis buffer containing 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, and 0.2% sodium azide in PBS, as described previously²¹. Collagenase and elastase activities in abdominal aortas were determined using EnzChek gelatinase/collagenase assay kit (Molecular Probes). Assays were performed at room temperature in 200 ul reaction buffer containing 20 ul of DQ collagen I (D-12060, Molecular probe), 20 ul of DQ collagen IV (D-12052, Molecular probe), or 50 ul of DQ elastin (provided by the kit) and 100 ul of the protein extracts at 200 ug/ml. Substrate hydrolysis was monitored at 0, 2, 4, and 6 hours at room temperature and fluorescence was detected in a fluorescence microplate reader (λ_{exc} =485 nm; λ_{em} =538 nm; with a lower cut-off at 515 nm). Background fluorescence, reflecting non-enzymatic hydrolysis, was subtracted and each value was normalized for the baseline fluorescence at 0 h. The proteolytic activity in the samples was derived directly from the initial slope and expressed as arbitrary units/hour (AU/hour).

Fluorescence-activated cell sorting (FACS)-analysis

ApoE^{-/-} mice were treated with Ang II using osmotic minipumps as described above. After 4 weeks, mice were sacrificed. Splenocytes were isolated to make single-cell

suspensions and analyzed using FACS CantoII (Becton Dickinson) flow cytometer. Erythrocytes in peripheral blood and spleen were removed by hypotonic lysis with NH_4Cl . Cells were first incubated with anti-CD16/32 (eBioscience) to block Fc receptor binding to antibodies in macrophages, neutrophils and mast cells and stained with anti-CD3-FITC, anti-CD8-Pacific blue, anti-CD25-APC (eBioscience) and anti-CD4-PerCp (BD-Biosciences Pharmingen, San Diego, CA). Foxp3-positive cells were detected with PE anti-mouse/rat Foxp3 Staining Set, according to the manufacturer's instruction (eBioscience). Peripheral blood leukocytes were further incubated with anti-CD11b-pacific blue (eBioscience, San Diego, CA) and anti-ly6G-pacific blue (BD-Biosciences Pharmingen) to detect monocytes (CD11b+ly6G-) and granulocytes (CD11b+ly6G+).

Statistical analysis

Values are expressed as mean $\pm$ SEM and a Mann-Whitney non-parametrical test was used to compare individual groups of animals. Probability values of < 0.05 were considered significant.

Results

Cathepsin K, S and C expression is elevated in AAA lesions but unaltered in Ang-II stimulated macrophages

CatK, S and C mRNA expression was unchanged in abdominal aortas of apoE^{-/-} mice (n=3, figure 7.1A), but sharply increased in AAA tissue of Ang-II infused apoE^{-/-} mice (n=3, figure 7.1A). In order to study if the upregulated cathepsin expression is attributable to Ang II exposure itself, independent of the underlying vasculopathy, catK, S and C mRNA expression was determined in bone marrow-derived macrophages from apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice after in vitro stimulation with 2.5 mM Ang II for 24 hours. Ang II exposure in vitro did not alter catK, S and C mRNA expression in apoE^{-/-} macrophages (figure 7.1B), nor did it differentially affect catS and C expression in catK^{-/-}/apoE^{-/-} macrophages (figure 7.1B) (n=6 in each group). Therefore, Ang II treatment by itself can not be held accountable for the induced catK, S and C mRNA expression in aneurysm tissue, suggesting that in this AAA model, cathepsin dysregulation is a direct consequence of aneurysm formation.

Cathepsin K deficiency did not protect against Ang II-induced aneurysm formation

Infusion of Ang II for 4 weeks induced AAA formation in 74% (14 out of 19, n=19 mice) of the apoE^{-/-} and 75% (15 out of 20, n=20 mice) of the catK^{-/-}/apoE^{-/-} mice. 5 mice from both apoE^{-/-} and catK^{-/-}/apoE^{-/-} did not show any aneurysm formation. Figure 7.2A shows the survival curve of mice before sacrificing, indicating that apoE^{-/-} and catK^{-/-}/apoE^{-/-} had similar mortality rates. Mortality in all cases was attributable to AAA rupture. 47% of apoE^{-/-} mice (9 out of 19) had non-fatal and 26% (5 out of 19)

Figure 7.1A CatK, S and C mRNA expression in AAA of Ang-II infused apoE^{-/-} mice and in nondiseased abdominal aorta of untreated apoE^{-/-} mice were measured by Q-PCR (relative expression values are normalized for cyclophilin expression). Figure 7.1B CatK, S and C mRNA expression in bone marrow-derived macrophages from apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice after incubation for 24 h with and without Ang II (2.5 μ M) were measured by Q-PCR (relative expression values are normalized for cyclophilin expression). *P=0.05.

had fatal rupture (death caused by AAA rupture). Similarly, of catK^{-/-}/apoE^{-/-} mice, 45% (9 out of 20) had a non-fatal and 30% (6 out of 20) a fatal rupture (figure 7.2B). The AAAs observed in both groups were mostly type III (figure 7.2C). CatK deficiency did not change the pathological score of the AAA (figure 7.2D). There was no difference between apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice regarding AAA lesion area as shown in figure 7.2E ($1.04 \times 10^6 \mu\text{m}^2 \pm 0.25 \times 10^6 \mu\text{m}^2$ versus $1.97 \times 10^6 \mu\text{m}^2 \pm 0.59 \times 10^6 \mu\text{m}^2$; n=10 for each group). All AAAs in catK^{-/-}/apoE^{-/-} mice and 86% of AAAs in apoE^{-/-} mice displayed complete medial breaks that resulted in marked dilation of the lumen (figure 7.2F, left panel). Both groups displayed media thickening (figure 7.2F, right panel). Full color figures are shown on page 173.

Cathepsin K deficiency altered collagen but not inflammatory cell content of AAA lesions

In the aneurysm area, Mac3⁺ macrophage ($0.8 \pm 0.3\%$ versus $1.0 \pm 0.3\%$, p=0.48, n=5-6) and CD45⁺ leukocyte content ($0.4 \pm 0.1\%$ versus $1.0 \pm 0.4\%$, p=0.199, n=5-6) did not differ between apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice (figure 7.3A and B). We also did not observe any significant differences in adventitial Mac3⁺ macrophage content of AAA lesions of apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice ($0.03 \pm 0.01\%$ versus $0.21 \pm 0.10\%$, respectively; p=0.27, n=5-6) (figure 7.3A). The number of CD45⁺ leukocytes tended to be increased in the adventitia of catK^{-/-}/apoE^{-/-} compared with apoE^{-/-} mice ($1.5 \pm 0.7\%$ versus $2.8 \pm 0.5\%$, respectively; p=0.068, n=5-6) (figure 7.3B). Lesional Ly-6G⁺ granulocytes were rather scarce in AAA. The relative number of Ly-6G⁺ granulocytes

Figure 7.2 Cathepsin K deficiency did not protect against Ang II-induced aneurysm formation. (A) Kaplan-Meier survival curve of mice. Premature death could in all cases be attributed to AAA rupture. (B) CatK deficiency did not affect the incidence of fatal and non-fatal rupture of catK^{-/-}apoE^{-/-} and apoE^{-/-} mice following treatment with Ang II. (C and D) CatK deficiency did not alter the severity of AAA as measured by pathology categorization (C) and average pathological score (D). CatK deficiency did not affect aneurysm size (E). Representative photographs showing EvG staining of aneurysms in apoE^{-/-} and catK^{-/-}apoE^{-/-} mice. Both groups displayed complete medial breaks and subsequent lumen expansion (F, left panel) and adventitial thickening (F, right panel). Full color figures are shown on page 173.

in the aneurysm area ($0.99 \pm 0.79 \times 10^{-5} / \mu\text{m}^2$ versus $0.08 \pm 0.06 \times 10^{-5} / \mu\text{m}^2$, $p=0.372$, $n=6$) was not significantly different between apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice (figure 7.3C). The average number of medial elastin breaks did not differ between apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice (20.9 ± 3.4 ($n=8$) versus 26.0 ± 2.7 ($n=10$), respectively; $p=0.062$) (Figure 7.3D/E).

Collagen content increased significantly in the aneurysm area of catK^{-/-}/apoE^{-/-} mice ($0.7 \pm 0.4\%$ versus $4.0 \pm 1.4\%$, $p=0.04$, $n=5$ and 7 , respectively) (figure 7.3F). However, there was no significant difference in medial collagen content between apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice ($58.2 \pm 0.9\%$ versus $60.5 \pm 3.6\%$, $p=0.74$; $n=10$ for each group) (Figure 7.3G). Representative pictures from Sirius red staining of AAA lesions from catK^{-/-}/apoE^{-/-} and apoE^{-/-} animals were shown in figure 7.3H. Full color figures are shown on page 173. Thus, catK deficiency increased aneurysm but not medial collagen content.

CatS and catC positive cells were higher in catK^{-/-}/apoE^{-/-} versus apoE^{-/-} mice

As catS⁺ cells were mainly present in the adventitia, analysis was confined to this location. Surprisingly, the percentage of catS⁺ cells in the adventitia in catK^{-/-}/apoE^{-/-} was significantly increased compared with apoE^{-/-} mice ($1.1 \pm 0.3\%$ versus $3.8 \pm 0.8\%$; $p=0.007$, $n=7$ and 6 , respectively) (figure 7.4A). Likewise, we observed more catC⁺ cells in the adventitia of AAA lesions of catK^{-/-}/apoE^{-/-} than in that of apoE^{-/-} mice ($0.02 \pm 0.02\%$ versus $0.2 \pm 0.10\%$, $p=0.013$; $n=6$ for each group) (figure 7.4B). The percentage of catC⁺ cells in the aneurysm area of apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice was similar (0% versus $0.04 \pm 0.03\%$, $p=0.14$, $n=6$ for each group) (figure 7.4B). Representative pictures of catS staining from catK^{-/-}/apoE^{-/-} and apoE^{-/-} mice are shown in figure 7.4C. Representative pictures of catC staining in adventitia and in aneurysm area from catK^{-/-}/apoE^{-/-} and apoE^{-/-} mice are shown in figure 7.4D and E, respectively. In order to determine cathepsin producing cells, serial sections were stained with antibodies against catS, catC, α -SMA and CD45. The catS-positive and catC-positive cells were mainly SMCs and leukocytes as shown in figure 7.5A for catK^{-/-}/apoE^{-/-} mice and 7.5B for apoE^{-/-} mice.

Cathepsin K deficiency did not change aortic proteolytic activity

The collagenolytic and elastinolytic activities in AAA lysates were determined using EnzChek Gelatinase/Collagenase Assay kit, as shown in figure 7.6 ($n=6$ for apoE^{-/-}, $n=7$ for catK^{-/-}/apoE^{-/-}). Cat K deficiency did not influence collagen type I, IV and elastin degradation in AAA lysates (figure 7.6A). As a positive control, assay of the degradation of collagen I, IV and elastin in the presence of collagenase and elastase provided by the EnzChek Gelatinase/Collagenase Assay Kit was shown in figure 7.6B.

Cathepsin K deficiency with Ang II infusion increased granulocyte population and

H

Figure 7.3. CatK deficiency altered collagen but not inflammatory cell content of AAA lesions. (A, B and C) CatK deficiency did not affect macrophage, leukocyte and neutrophil content in AAA. The number of Mac3-positive (A) and CD45-positive (B) cells in the aneurysm area and in the adventitia were corrected for the total number of cells in the entire aneurysm lesion and adventitia respectively. The Ly-6G+ cell density in the aneurysm area was obtained by calculating the number of LyC6+ cells per lesion area (C). (D and E) The average number of elastin breaks in media was not significantly different between catK-/-/apoE-/- and apoE-/- mice (D). Representative pictures of elastin breaks in AAA lesions from catK-/-/apoE-/- and apoE-/- mice are shown in E. Arrows point to full transmural breaks and arrowheads point to focal breaks of a single elastic lamina. (F and G) CatK deficiency increased aneurysm (F; *p=0.04) but not medial collagen content (G). Representative pictures of Sirius red stainings of AAA lesions from catK-/-/apoE-/- and apoE-/- animals were shown in figure 7.3H. Full color figures are shown on page 173.

activated T cell numbers

FACS was performed to study the systemic effect of catK deficiency in untreated (n=7 for apoE-/-, n= 8 for catK-/-/apoE-/- mice) versus Ang II-infused (n=7 for each group) apoE-/- mice. CatK deficiency in Ang II-infused mice did not influence CD3+ T cell, Foxp3+ regulatory T cell and monocyte (CD11b+Ly6G-) population in blood and spleen (data not shown). Surprisingly, granulocyte (CD11b+Ly6G+) counts in blood were significantly increased in catK-/-/apoE-/- compared with apoE-/- mice upon Ang II infusion (Figure 7.7A). Moreover, in Ang II-infused mice with catK deficiency, the number of effector T-helper cells in blood as manifested by the relative abundance of CD4+CD25+FoxP3- within the CD4+ significantly enhanced (Figure 7.7B). However, such effects were not observed in mice without Ang II infusion (Figure 7.7A and B).

Discussion

AAA is a life-threatening vascular disease characterized by chronic inflammation, excessive degradation of the ECM, and increased expression of proteases such as MMPs and cathepsins^{8, 22}. In view of the strong elastolytic and collagenolytic activity of catK and its elevated expression in human AAA lesions, we hypothesized that a deficiency of this cysteine protease would decrease AAA formation in Ang II-infused apoE-/- mice. In line with the human data, catK, S and C were all shown to be strongly upregulated in AAA in Ang II-infused apoE-/- mice compared with nondiseased abdominal aorta

Chapter 7

Figure 7.4. CatK deficiency led to enhanced presence of catS+ (A) and catC+ cells (B) in aneurysms. Expressed is the relative catS+ cell content in the adventitia of aneurysms calculated by dividing the number of catS+ cells by the total number of cells in adventitia (A; *P=0.007). Representative pictures of catS staining from catK-/-/apoE-/- and apoE-/- mice are shown in C. The relative catC+ cell content in adventitia (*p=0.013) or aneurysm (p=0.14) was calculated as described above (B). Representative pictures of catC staining in adventitia and in aneurysm area from catK-/-/apoE-/- and apoE-/- mice are shown in D and E, respectively.

B

apoE^{-/-}

Figure 7.5. Representative micrographs of catS, catC, α-SMA and CD45⁺ stained serial sections of adventitia of catK^{-/-}/apoE^{-/-} (A) and apoE^{-/-} mice (B). CatS⁺ or catC⁺ cells that are also positive for α-SMA or CD45 are encircled.

Figure 7.6. CatK deficiency does not lead to altered collagen I, IV and elastin degradation in aneurysm lysates between catK-/-/apoE-/- mice and apoE-/- mice as measured by fluorometric enzyme (A). As a positive control, proteolytic activity was measured upon addition of reference enzymes of collagenase and elastase, both at 0.02U/well (B).

Figure 7.7. Flow cytometry analysis of circulating T cells and granulocytes. CatK deficiency significantly increased circulating granulocyte percentage (A) as well as the relative abundance of CD4+CD25+FoxP3- within the CD4+ population (B) in Ang II infused apoE-/- mice. However catK-/-/apoE-/- did not alter granulocyte percentage (A) and CD4+CD25+FoxP3- percentage of CD4+ cells (B) in non-infused apoE-/- mice. *p<0.05

from untreated apoE-/- mice, confirming the validity of our model to study the effects of catK deficiency on AAA formation. Interestingly, macrophage catK, S and C expression was not affected by Ang II stimulation, suggesting that catK, S and C upregulation is associated with the pathophysiology of AAA rather than with Ang II treatment per se. Unexpectedly, despite a strong upregulation of catK in Ang II-induced AAA in apoE-/- mice, catK deficiency did not reduce aneurysm formation nor did it reduce aneurysm size, severity, and elastic lamina degradation. We demonstrate that the lack

of protective effect of catK deficiency against aneurysm formation might at least in part be explained by a compensatory activation of other cysteine proteinases in the vascular wall. Punturieri, et al has demonstrated that macrophages from catK-deficient patients (compound heterozygous), maintained full elastolytic potential by mobilizing catL and catS but not MMPs²³. Our finding of similar medial elastin breaks and proteolytic activity in AAA lysates of catK-/-/apoE-/- mice and apoE-/- mice corroborates this notion. Furthermore, catS⁺ and catC⁺ cells were more abundantly present in the aneurysm area of catK-/-/apoE-/- mice compared with apoE-/- mice, again confirming proteolytic activity in catK deficient animals is compensated by other cysteine proteinases. In an earlier study, we determined gene expression profiles of aortic arches of catK-/-/apoE-/- and apoE-/- animals. A closer look at gene expression profiles of aortic arches of catK-/-/apoE-/- versus apoE-/- mice, showed that the major MMPs (MMP 1, 2, 7, 9, 11, 14 and 23) in aortic tissue were not differentially expressed between the two groups²⁴.

The role of inflammatory cells in AAA formation has been intensively studied^{25, 26}. Granulocytes are considered to be critical in the formation of AAA, as granulocyte depletion was seen to inhibit AAA development associated with attenuated inflammatory cell recruitment²⁷. T cells may also play a significant role in AAA formation as aneurysms in patients have substantial proportions of identical β -chain TCR transcripts²⁸. These findings suggest that active inflammation is present in AAA. Therefore, the effects of catK deficiency were further studied at systemic level in catK-/-/apoE-/- and apoE-/- mice that had or had not received Ang II infusion. Surprisingly, there was a dramatic increase in the granulocyte and effector T cell population in catK deficient mice after Ang II infusion, which is consistent with the increased adventitial leukocyte content in catK-/-/apoE-/- compared to apoE-/- mice observed in our study. Strikingly, the above mentioned systemic effects were not observed in mice without Ang II infusion. The results suggest that the effects of catK deficiency on the hematopoietic system only manifest under conditions of Ang II induced inflammation. In an earlier study, we observed decreased plaque progression and atherosclerotic plaque area in catK-/-/apoE-/- compared with apoE-/- mice¹⁶. In addition, the number of elastin breaks in the media underlying the atherosclerotic plaque was substantially decreased in advanced lesions of catK-/-/apoE-/- compared with apoE-/- mice. In contrast, this study showed that catK deficiency in apoE-/- mice did not confer protection against AAA formation, and did not reveal any effect on the number of elastin breaks and on proteolytic activity in AAA lysates. Unlike the atherosclerosis study, which was performed in non-infused apoE-/- mice, here apoE-/- mice were infused with Ang II to induce AAA. Combined with the observed pro-inflammatory effects of catK deficiency on circulating leukocyte subsets, which were strictly Ang II dependent as well, this raises the intriguing option that the observed pro-inflammatory effects of catK deficiency are opportune only in Ang II infused apoE-/- mice.

In conclusion, although catK has strong elastinolytic and collagenolytic activity, both of which are regarded critical processes in AAA, its deficiency surprisingly did not protect against aneurysm formation. Our data suggest that the apparent lack of effects of catK deficiency on medial elastin breaks and on elastinolytic and collagenolytic activity in AAA lysates may point to a compensatory increase in proteolytic activity exerted by other ECM degrading enzymes, such as catS and catC in the aneurysm. Although catK deficiency did increase aneurysm collagen content, it clearly was unable to reverse aneurysm formation or severity. In addition, catK deficiency was associated with an unexpected increase in the number of circulating granulocytes and effector T-helper cells, which might counteract the potentially protective pro-fibrotic effect of catK deficiency in AAA. These data are remarkable given the previously reported deleterious effects of this protease in atherosclerosis and point to fundamental differences in the proteolytic processes that underlie AAA and atherosclerosis.

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Chapter 7

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8

Cathepsin K deficiency reduces carotid artery remodeling due to formation of a macrophage-rich lesion in apoE^{-/-} mice, but does not affect macrophage-poor lesion formation in wild type mice

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Abstract

Cathepsin K (catK) is a potent lysosomal cysteine protease involved in extracellular matrix (ECM) degradation and inflammatory remodelling responses. Here we have investigated the contribution of catK deficiency on carotid arterial remodeling in response to flow cessation in apoE^{-/-} and wild type (WT) background.

CatK protein expression was significantly increased in carotid artery ligation-induced intimal lesions in apoE^{-/-} compared with WT mice, suggesting that catK contributes differently in intimal hyperplasia in apoE^{-/-} and WT mice. In keeping with data on previous studies, ligation-induced hyperplasia was more pronounced in apoE^{-/-} compared to WT mice. Surprisingly, catK deficiency completely blunted the augmented hyperplastic response to flow cessation in apoE^{-/-}, while having no effect at all on vascular remodelling in WT mice. As catK deficiency did not markedly alter lesion collagen content and elastic laminae fragmentation *in vivo*, nor macrophage elastolytic and collagenolytic activity *in vitro*, we focused on effects of catK on (systemic) inflammatory response. CatK deficiency significantly reduced circulating CD3 T-cell numbers, but increased the regulatory T cell subset in apoE^{-/-} but not WT mice. Moreover, catK deficiency changed CD11b+Ly6G-Ly6C^{high} monocyte distribution in apoE^{-/-} but not WT mice and tended to bias macrophage polarization towards a M2a differentiation. In conclusion, catK deficiency almost completely blunted the increased vascular remodeling response of apoE^{-/-} mice to flow cessation, possibly by correcting hyperlipidemia-associated pro-inflammatory effects on peripheral immune response.

Introduction

CatK is the most potent mammalian elastase of the cathepsin family of proteases yet known and possesses collagenolytic as well as elastinolytic activity^{1, 2}. Next to its involvement in ECM degradation, catK was reported to modulate inflammatory responses via the TGF- β ³ and TLR-9⁴ signaling pathway. In a previous study, apoE^{-/-} mice with a targeted disruption of the catK gene showed reduced atherosclerotic lesion growth³. In addition, uptake of modified lipoproteins was enhanced in catK^{-/-} macrophages, potentially via a caveolin-1- and scavenger receptor CD-36- dependent pathway^{3, 5}. Selective disruption of leukocyte catK was seen to dramatically decrease collagen and increase macrophage content of atherosclerotic lesions, leaving lesion size unaffected. It was suggested that smooth muscle cell (SMC) migration was hampered due to the abolished elastinolytic activity of macrophage catK⁶. Clearly both these studies suggested a role for macrophage catK in atherosclerosis.

In the vascular remodeling process, proliferation and migration of SMC from the media to the intima contribute to intima formation^{7, 8}. Migration of SMCs requires degradation of ECM. In balloon-injured rat carotid arteries, increased mRNA and protein levels of catK were found to be enhanced in the neointima⁹. There was a significant increase in elastolytic and collagenolytic activity in the intima tissue extracts compared with uninjured control vessels⁹. The enhanced ECM degrading potential and the increased expression and activity of catK during neointima formation suggest that catK may participate in the remodeling processes during neointima formation. To further study the potential role of catK in vascular remodeling, we compared carotid artery remodeling in the flow cessation model in both the catK-deficient apoE^{-/-} background and catK-deficient WT background (catK^{-/-} /apoE^{-/-} vs apoE^{-/-} and catK^{-/-} vs C57Bl6). In this model, flow cessation is used to accelerate the formation of macrophage-rich neointima lesions in hypercholesteremic apoE-deficient mice and the formation of a macrophage-poor neointima lesion in the WT mice¹⁰.

We found that catK deficiency significantly reduced macrophage-rich neointima formation in the hyperlipidemic apoE^{-/-} mice. However, macrophage-poor neointima formation in C57Bl6/WT mice was unaffected. Furthermore, catK deficiency significantly reduced circulating CD3 T-cell numbers, but increased the regulatory T cell subset in apoE^{-/-} but not WT mice, suggesting that catK deficiency protected against macrophage-rich lesion formation possibly by correcting hyperlipidemia-associated pro-inflammatory effects on peripheral immune response.

Materials and Methods

Experimental model of flow cessation induced intimal hyperplasia/vessel retraction

Chapter 8

In this study, flow cessation was induced in C57Bl6, catK^{-/-}, apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice. Male apoE^{-/-} mice on a C57Bl6/J background were obtained from IffaCredo (Lyon, France). CatK^{-/-} mice, kindly provided by Dr P Saftig, were generated on an outbred 129SVJ-C57Bl/6J genetic background¹¹. We subsequently backcrossed the catK^{-/-} mice at least 9 times with C57Bl6/J mice and apoE^{-/-} mice to generate catK^{+/+} and catK^{-/-} mice on a C57Bl6/J (WT) or apoE^{-/-} background, respectively. Animals were maintained in accordance with the Dutch government guidelines and animal experiments were approved by the regulatory authority of the University of Maastricht.

Macrophage-rich and -poor intimal lesions were induced by unilateral flow cessation of the common carotid artery in hypercholesterolemic apoE^{-/-} and WT mice, respectively as described by Ivan et al¹⁰. WT and catK^{-/-} mice from C57Bl6/J background (17-18 weeks of age) were fed a normal chow (SNIFF, V1534) throughout the experiment. In brief, mice were anesthetized with 2.5% isoflurane, and the common right carotid artery was ligated with a silk suture (5-0) near the bifurcation. The same procedure was pursued for apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice (13-16 weeks of age), at which mice were fed a western type diet containing 0.25% cholesterol (Research Diet, diet number 4021.06) from two weeks before ligation onwards^{10, 12}.

Tissue harvesting and analysis

Four weeks after ligation, mice were sacrificed. The arterial tree was perfused with PBS containing 0.1 mg/ml nitroprusside (Sigma) and subsequently with 1% paraformaldehyde via a catheter inserted into the left cardiac ventricle. Right carotid artery cross-sections (4 µm thick) were cut at 100 µm intervals. At each level of 100 µm, a cross-section was stained with Lawson solution and used to determine intima, lumen and total vessel area as well as elastic lamina fragmentation. The relative intimal collagen content, i.e. the percentage of total intima area that stained positive for Sirius red, was determined under a microscope coupled to a computerized morphometry system (Quantimet 570, Leica). Morphometric analyses were performed by one blinded investigator (L Bai, intra-observer variability was < 10%) using a computerized morphometry system (Quantimet 570, Leica). Serum cholesterol level was determined with the CHOD-PAP method (Roche Diagnostics).

Immunohistochemistry

Immunohistochemical stainings were performed to determine macrophage (anti-Mac3), T cell (anti-CD3+) and SMC (α-smooth muscle actin, α-SMA) content, as described previously⁵. The relative intimal macrophage, T cell and SMC content, was calculated

by dividing the number of lesional Mac3+, CD3+ and α -SMA+ cells, respectively by the total number of cells in the lesion. Expression of catK was determined using a goat anti-human catK (c16) antibody (1:100, Santa Cruz). The relative catK-positive area was determined by a computerized morphometry (Quantimet 570, Leica). Mouse spleen was used as positive control for CD3 and catK staining, and mouse atherosclerotic lesions as positive control of both Mac3 and α -SMA staining. Deletion of the primary antibody served as negative control. No positive staining for any of the antibodies was observed on negative controls.

All measurements were conducted by a single investigator (L Bai, intra-observer variability was < 10%).

Fluorescence-activated cell sorting (FACS)-analysis

Splenocytes and blood were isolated from apoE^{-/-}, catK^{-/-}/apoE^{-/-}, WT, and catK^{-/-} mice. Erythrocytes in peripheral blood and spleen were removed by hypotonic lysis with NH₄Cl. Cells were incubated first with anti-CD16/32 (eBioscience) to block Fc receptor binding to antibodies on macrophages, neutrophils and mast cells and stained with anti-CD3-FITC, anti-CD8-Pacific blue, anti-CD25-APC, anti-CD45R(B220)-Pe-Cy7 (eBioscience) and anti-CD4-PerCp (BD-Biosciences Pharmingen). Foxp3-positive cells were detected with PE anti-mouse/rat Foxp3 Staining Set, according to the manufacturer's instruction (eBioscience). Peripheral blood leukocytes were incubated with anti-CD11b-pacific blue (eBioscience) and anti-ly6G-PE (BD-Biosciences Pharmingen) to detect monocytes (CD11b⁺ly6G⁻) and granulocytes (CD11b⁺ly6G⁺). For measurement of inflammatory monocytes (CD11b⁺Ly6G⁻Ly6C^{high}) cells were stained with anti-Ly6C-FITC (Miltenyi Biotec). Blood natural killer (NK) cells were stained by anti-CD49b(DX5)-APC (Miltenyi Biotec).

Cell isolation and culturing

Bone marrow-derived macrophages (BMM) were isolated from the femur and tibia of WT, catK^{-/-}, apoE^{-/-}, and catK^{-/-}/apoE^{-/-} mice. Cells were cultured in standard RPMI culture medium containing L-glutamine, HEPES, 10% fetal calf serum, 100 IU/mL penicillin/streptomycin, and 15% L929 cell conditioned medium. Macrophage polarization was induced by culturing BMM for 16 h at a density of 1x 10⁶ cells/ml in the aforementioned medium supplemented with 10 ng/ml LPS, to induce M1 polarization, or 20 ng/ml IL-4 to induce M2 polarization.

Quantitative RT-PCR

RNA was extracted from BMM lysate with Nucleospin RNA II kit (MACHEREY-NAGEL). cDNA was generated with iScript™ CDNA synthesis kit (BIO-RAD). Real-time PCR was done with a Taqman IQ™ SYBR Green Super Mix (BIO-RAD). Cyclophilin A was used to normalize RNA quantity. Primer sequences of arginase, MR (mannose receptor), IL-10, Nos2, IL-18, IFN- γ and cyclophilin are shown in table 8.1. The data were expressed as ratio of the quantity of specific transcripts to the quantity of the cyclophilin A gene. The relative gene expression data from WT, catK^{-/-}, apoE^{-/-} and catK^{-/-}/apoE^{-/-} background was then normalized to the data of WT background.

Table 8.1

	<i>Forward primer (5'-3')</i>	<i>Reverse primer (5'-3')</i>
Arginase	ATGGAAGAGACCTTCAGCTAC	GCTGTCTTCCCAAGAGTTGGG
MR	GCAAATGGAGCCGTCGTGTGC	CTCGTGGATCTCCGTGACAC
IL-10	TCTTACTGACTGGCATGAGGATCA	GTCCGCAGCTCTAGGAGCAT
Nos2	CAGCTGGGCTGTACAAACCTT	CATTGCAAGTGAAGCGTTTCG
IL-18	ACAACCTTGGCCGACTTCAC	GGGTTCACTGGCACTTTGAT
IFN- γ	TGGCTGTTTCTGGCTGTTACTG	GCTCTGCAGGATTTTCATGTCA
Cyclophilin	CAAATGCTGGACCAAACACAA	TTCACCTTCCCAAAGACCACAT

Elastase and collagenase activity assay

BMM were isolated from the femur and tibia of WT, catK^{-/-}, apoE^{-/-}, and catK^{-/-}/apoE^{-/-} mice. Cell lysates were extracted using cold RIPA buffer containing 25mM Tris HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS. Protein concentration in the samples was determined with BCA protein assay reagent kit (PIERCE). Collagenase and elastase activities in the cell lysates were determined using EnzChek gelatinase/collagenase assay kit (Molecular Probes). Assays were performed at room temperature in 200 μ l reaction buffer containing 20 μ l of DQ collagen I (D-12060, Molecular probe), 20 μ l of DQ collagen IV (D-12052, Molecular probe), or 50 μ l of DQ elastin (provided by the kit) and 100 μ l of the protein extracts at 200 μ g/ml. Substrate hydrolysis was monitored at 0, 1, 2, 3 and 4 hours at room temperature by fluorescence spectrometry in a fluorescence microplate reader (λ_{exc} =485 nm; λ_{em} =538 nm; with a lower cut-off at 515nm). Values were corrected for background fluorescence, reflecting non-enzymatic probe hydrolysis.

Statistics

Statistical analyses were performed using a nonparametric Mann–Whitney U test. Data are expressed as mean±SEM, and differences were considered statistically significant at $P<0.05$.

Results

Intimal lesions in apoE^{-/-} mice have increased catK protein expression

Figure 8.1 CatK expression in macrophage-rich (apoE^{-/-}) and macrophage-poor (WT) flow cessation-induced intimal lesions. A. Representative pictures of catK staining in WT and apoE^{-/-} mice. Full color figures are shown on page 174. B. Relative catK-positivity was calculated by dividing the anti-catK Ab stained area by the total intima area ($P=0.027$; $n=5$ for WT, $n=12$ for apoE^{-/-}).

CatK expression in flow cessation-induced intimal lesions of WT mice was very low and mainly confined to SMCs and the ECM ($n=5$). In contrast, macrophage-rich lesions of apoE^{-/-} mice showed pronounced expression of catK ($n=12$, figure 8.1B), primarily residing in cytoplasm of macrophages, but also in SMCs and, in a more diffuse manner, in the extracellular matrix (figure 8.1A). Full color figures are shown on page 174.

CatK deficiency attenuates flow cessation-induced intimal hyperplasia in apoE^{-/-} but not WT mice

CatK deficiency did not alter serum cholesterol level in neither apoE^{-/-} nor WT mice

(n=14-15 for apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice; n=7 for WT and catK^{-/-} mice, figure 8.2).

ApoE deficiency significantly enhanced lumen area ($p=0.027$), average lesion area ($p=0.006$) and total vessel area ($p=0.009$) compared to WT background, indicating a prominent outward remodeling in apoE^{-/-} background. Average lesion area, total vessel and lumen area were comparable in WT and catK^{-/-} mice (n=6-8, figure 8.3B). In sharp contrast, catK deficiency was found to protect against intimal hyperplasia in apoE^{-/-} mice. Average lesion and total vessel area were significantly reduced in catK^{-/-}/apoE^{-/-} compared to apoE^{-/-} mice (n=14-15, figure 8.3B), whereas lumen area was not significantly different (n=14-15, figure 8.3B). In fact, lesion size and total vessel area of catK^{-/-}/apoE^{-/-} mice were almost identical to that of normolipidemic WT and catK^{-/-} mice. Representative micrographs showing HE staining of intima lesions in WT, catK^{-/-}, apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice are shown in figure 8.3A. Full color figures are shown on page 174. Thus, apoE deficiency induced a prominent outward remodeling and neointimal growth in ligated right carotid artery compared to WT. CatK deficiency almost completely reversed this outward remodeling to basal levels of WT mice.

CatK deficiency did not alter lesion composition in apoE^{-/-} and WT mice

There was no difference in SMC content, collagen content and cell density in lesions of WT and apoE^{-/-} mice (figure 8.4A-C). Macrophage number (n=14-15, figure 8.4A), CD3 + T cell number (n=14-15, figure 8.4A) SMC number (n=13-15, figure 8.4A) as well as collagen content (n=14-15, figure 8.4B) did not differ between apoE^{-/-}/catK^{-/-} and apoE^{-/-} mice. Total cell number tended to be higher in macrophage-rich lesions of catK^{-/-}/apoE^{-/-} than of apoE^{-/-} mice (n=14-15, figure 8.4C). As cleaved caspase-3 positive cells were scant but present at essentially similar numbers in catK^{-/-}/apoE^{-/-} and apoE^{-/-} lesions (data not show), the increased cell density in catK^{-/-}/apoE^{-/-} mice is probably not attributable to a decrease in apoptotic rate. SMC, collagen content and total cell density of macrophage-poor lesions of WT and catK^{-/-} mice were comparable (n=6-8, figure 8.4A-C).

Despite the strikingly different effect of catK deficiency on intimal hyperplasia and the differential intimal catK expression in WT and apoE^{-/-} mice, catK deficiency did not affect medial elastic laminae fragmentation in both mouse strains (n=6-8 for WT background and 14 for apoE^{-/-} background, respectively; figure 8.5A), suggesting that catK is not a critical elastolytic protease in flow cessation induced elastic laminae breakdown.

Macrophage elastolytic activity is not markedly affected by catK deficiency in both

Figure 8.2. The absence of catK did not affect serum total cholesterol in both WT and apoE^{-/-} mice. Values represent the mean $\pm$ SEM. (n=14-15 for apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice; n=7 for WT and catK^{-/-} mice).

A

B

Figure 8.3 Effect of catK deficiency on intimal hyperplasia. A. Representative micrographs showing HE staining of intima lesions in WT, catK^{-/-}, apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice. Full color figures are shown on page 174. B. CatK deficiency did not affect lumen, intima and total vessel area in WT mice. CatK deficiency significantly reduced intima ($P=0.019$) and total vessel area ($P=0.024$) in apoE^{-/-} mice. Values represent the mean $\pm$ SEM. (n= 6-8 for WT and catK^{-/-} mice, n=14-15 for apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice).

Figure 8.4 CatK deficiency did not affect macrophage, CD3 and SMC content in the macrophage-rich lesions of apoE^{-/-} mice (A). CatK deficiency also did not alter SMC content in the macrophage-poor lesions of WT mice (B). The number of Mac3-positive, CD3-positive and α -SMA-positive cells in the intima area were corrected for the total number of cells in the entire intima area. CatK deficiency did not change collagen content in the intima area in apoE^{-/-} and WT mice (B). CatK deficiency did not significantly alter cell density in the

intima area in apoE^{-/-} and WT mice (C). Total cell number in neointima lesion was corrected for the corresponding lesion area. Values represent the mean $\pm$ SEM. (n= 6-8 for WT and catK^{-/-} mice, n=14-15 for apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice).

WT and apoE^{-/-} mice

Ivan et al have shown that macrophages increase expansive arterial remodeling and intimal hyperplasia by promoting matrix degradation¹⁰. Therefore, we studied the proteolytic activity of BMM isolated from catK^{-/-}/apoE^{-/-}, apoE^{-/-}, catK^{-/-}, and WT mice. Elastinolytic activity in apoE^{-/-} derived BMM was not affected by catK deficiency (figure 8.5B). Furthermore, collagen type I and collagen IV degradation in apoE^{-/-} derived BMM was unchanged in catK^{-/-} macrophages (figure 8.5C and D). Similarly, catK deficiency in WT BMM did not influence degradation of collagen type I, IV and elastin (figure 8.5E-G).

Peripheral immune activity was altered in catK deficient apoE^{-/-} mice but not in catK deficient WT mice

As outward remodeling was more pronounced in the single apoE^{-/-} mice, which is possibly mediated by intimal leukocytes, we investigated the inflammatory status of catK deficient mice in more detail. Peripheral immune activity was assessed by FACS

Figure 8.5 A: CatK deficiency did not affect medial elastin break frequency in apoE^{-/-} and in WT mice. Frequency is expressed as average number of breaks per cross-section. Elastin, collagen I and IV degradation activity was not significantly altered in BMMs of CatK^{-/-}/apoE^{-/-} mice compared to apoE^{-/-} mice (B-D). CatK deficiency in the C57Bl6 background also did not lead to altered collagen I, IV and elastin degradation activity in BMMs as measured by fluorometric enzyme (E-G). Values represent the mean $\pm$ SEM. (n=6 for each group).

analysis. There was no difference in CD3⁺ T cells (expressed as percentage of total living cells) in spleen and blood between WT and apoE^{-/-} mice. However, apoE^{-/-} significantly enhanced natural killer (NK) cells (expressed as percentage of total living cells) in blood compared with WT mice ($p=0.0041$). Furthermore, apoE^{-/-} dramatically reduced the percentage of regulatory T cells (CD4⁺CD25⁺FoxP3⁺ relative to CD4⁺ T cells) in spleen ($p=0.0021$) and blood ($p=0.0012$) compared with WT mice. In addition, apoE^{-/-} mice showed significantly decreased level of inflammatory monocytes (CD11b⁺Ly6G⁺Ly6C^{high}) in blood compared with WT mice. Thus apoE^{-/-} mice showed increased levels of circulating NK cells and decreased regulatory T cell and inflammatory

monocyte numbers compared with WT mice.

CD3⁺ T cells in spleen and NK cells in blood decreased in catK^{-/-}/apoE^{-/-} mice compared with apoE^{-/-} mice (n=6, figure 8.6A). While the total CD3 T cell content of spleen was reduced in catK^{-/-}/apoE^{-/-} mice, the percentage of regulatory T cells (CD4⁺CD25⁺FoxP3⁺ relative to CD4⁺ T cells) was significantly increased in blood of catK^{-/-}/apoE^{-/-} mice (n=6, figure 8.6B). This is consistent with an increase in splenic regulatory T cells in catK^{-/-}/apoE^{-/-} mice (n=6, figure 8.6B). Thus, catK deficiency normalizes regulatory T cell levels in apoE^{-/-} mice to levels found in WT. Although evidence implicating the direct role of regulatory T cell on vascular remodeling is lacking, regulatory T cell deletion significantly augmented post-ischemic neovascularization¹³. These effects were correlated with enhanced accumulation of CD3⁺ T cells in the ischemic leg¹³. On the other hand, treatment of CD28^{-/-} mice with anti-CD3⁺ antibody enhanced the number of endogenous Treg cells and led to a significant reduction of the postischemic inflammatory response and neovascularization¹³. Thus, regulatory T cell might inhibit outward remodeling through abrogating inflammatory responses.

Interestingly, there was a significant increase in the percentage of inflammatory monocytes (CD11b⁺Ly6G⁺Ly6C^{high}) in blood of catK^{-/-}/apoE^{-/-} compared with apoE^{-/-} mice (n=6, figure 8.6C). Consistently, the percentage of CD11b⁺Ly6G⁺Ly6C^{high} cells of monocytes was significantly reduced in catK^{-/-}/apoE^{-/-} relative to apoE^{-/-} mice (n=6, p=0.047, figure 8.6C). In contrast, catK deficiency in normolipidemic C57Bl6 mice did not affect peripheral immune profiles (n=6, figure 8.6D, E and F). Thus, catK deficiency in apoE^{-/-} mice dramatically reduced systemic CD3⁺ and NK cell counts and an increase in Treg cell population, which in fact were normalized to base levels of WT mice, whereas no effects on these cell populations were seen in the WT mice. Based on the above findings, the systemic anti-inflammatory potential of catK deficiency in apoE^{-/-} mice might explain the observed protective effect of catK deficiency against macrophage-rich lesion formation as apoE^{-/-} mice develop a macrophage-rich neointima after carotid artery ligation that is characterized by stronger inflammatory responses compared to WT background¹⁴

CatK deficiency tended to shift non-stimulated apoE^{-/-} BMM to M2a phenotype

Several studies have shown that macrophage-derived inflammatory growth factors contribute to intimal lesion enlargement^{15, 16,17}. CatK deficiency has been shown to affect macrophage foam cell formation⁵, however the effect of catK on macrophage polarization is unknown. We therefore studied the effect of catK deficiency on macrophage polarization in vitro. The expression of M2 macrophage markers such

Figure 8.6 Flow-cytometric analysis of the effect of catK deficiency on CD3⁺, NK, CD4⁺CD25⁺Foxp3⁺ regulatory T cells and CD11b⁺Ly6G⁺Ly6C^{high} monocytes in spleen and blood. Percentage of CD3⁺ T cells in spleen (P=0.025) and NK cells in blood (P=0.025) were significantly reduced in catK^{-/-}apoE^{-/-} mice compared with apoE^{-/-} (A). The regulatory T cell population defined as CD4⁺CD25⁺Foxp3⁺ cells, was significantly increased in catK^{-/-}apoE^{-/-} mice in blood (B, P=0.025). CD11b⁺Ly6G⁺Ly6C^{high} monocytes were significantly increased (P=0.035) and CD11b⁺Ly6G⁺Ly6C⁻ monocytes significantly reduced (P=0.047) in blood of catK^{-/-}apoE^{-/-} compared with apoE^{-/-} mice (C). CatK deficiency in the WT mice did not affect peripheral immune activity (D, E and F). Values represent the mean $\pm$ SEM. (n=6 for each group).

as arginase-1 (marker for M2a macrophage), IL-10 (marker for M2b macrophage), mannose receptor (MR, marker for M2a), and M1 macrophage markers such as IL-18, Nos2 and IFN- γ were measured in BMM from catK-/-/apoE-/-, apoE-/-, catK-/-, and WT mice without prior stimulation to assess baseline polarization. Compared to WT, apoE-/- significantly enhanced both M1 macrophage marker IL-18 ($p=0.006$) and M2 macrophage marker arginase ($p=0.004$), MR ($p=0.037$) and IL-10 ($p=0.055$). However, Nos2 expression was significantly reduced in apoE-/- mice compared to WT ($p=0.004$). CatK deficiency in WT mice did not markedly alter macrophage polarization. Compared to WT, catK-/- BMM only showed a tendency towards enhanced arginase expression ($n=6$, figure 8.7A). Furthermore, the expression of IL-10, MR, Nos2 and IL-18 were not different between catK-/- and WT -derived BMM ($n=6$, figure 8.7A). The expression of IFN- γ was below detection limit. In sharp contrast, BMM-derived from catK-/-/apoE-/- mice displayed a significantly decreased expression of IL-10 ($n=6$, figure 8.7B), while conversely arginase expression was increased compared with BMM-derived from apoE-/- mice ($n=6$, figure 8.7B). In addition, a tendency to a decreased expression of the M1 macrophage marker of IL-18 was found in catK-/-/apoE-/- BMM compared with apoE-/- BMM ($p=0.078$, $n=6$, figure 8.7B). There was no difference in Nos2 and MR expression between catK-/-/apoE-/- and apoE-/- -derived BMM ($n=6$, figure 8.7B). Again, the expression of IFN- γ by non-stimulated cells was below detection limit. Therefore, deficiency of catK in the apoE-/- background skewed basal macrophage polarization towards a M2a macrophage phenotype.

Cytokine induced macrophage polarization towards M2 and M1 is amplified in catK deficient apoE-/- bone marrow macrophages

We next analyzed the capacity of BMM to polarize in response to IL-4 (M2) or LPS (M1) in vitro. CatK deficient macrophages showed amplified response to polarizing cytokines. CatK deficiency in WT mice did not affect M2 marker expression upon IL-4 stimulation ($n=6$, figure 8.7C). However catK deficiency significantly enhanced LPS-induced IL-18 and Nos2 expression ($n=6$, figure 8.7D). Therefore, catK deficiency in WT mice skewed macrophage polarization towards a M1 phenotype.

Upon stimulation with IL-4, catK-/-/apoE-/- -derived BMM tended to increase both arginase-1 ($n=6$, figure 8.7E) and MR expression ($n=6$, figure 8.7E) compared with apoE-/- -derived BMM. There was no difference in IL-10 expression between catK-/-/apoE-/- and apoE-/- BMM upon IL-4 stimulation ($n=6$, figure 8.7E), while that of IL-4 was below detection limit for all the samples. Intriguingly, LPS stimulation led to increased induction of NOS2 and to a lesser extent IL-18 in catK-/-/apoE-/- versus apoE-/- - BMM ($n=6$, figure 8.7F). There was no difference in LPS-induced IFN- γ expression between the two groups. Thus, catK deficiency in apoE-/- mice enhanced the cytokine

Figure 8.7 Effect of catK deficiency on M1 and M2 macrophage marker expression of non-stimulated and IL-4/LPS stimulated BMM. BMM from WT, catK^{-/-}, apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice were isolated and cultured in the absence or presence of LPS/IL-4 for 16 h. BMM cell lysates were analyzed for M1 and M2 marker expression. CatK^{-/-} did not significantly change M1 and M2 marker expression in BMM (A). CatK^{-/-}/apoE^{-/-}-derived BMM increased arginase expression (P=0.004) and significantly decreased IL-10 expression (P=0.004) compared with apoE^{-/-}-derived-BMM (B). The deficiency of catK in WT mice significantly enhanced LPS-induced IL-18 expression without altering the expression of other M1/M2 markers (C and D). Upon stimulation with IL-4, catK^{-/-}/apoE^{-/-}-derived BMM did not significantly change both arginase and MR expression compared with apoE^{-/-} BMM (E). Nos2 expression was significantly increased in catK^{-/-}/apoE^{-/-}-derived BMMs upon LPS stimulation (p=0.004, F). Values represent the mean ± SEM. (n=6 for each group).

induced expression of both M2 and M1 macrophage marker, although the effect on IL-4-induced M2 macrophage marker expression was moderate and borderline significant.

Discussion

Vascular remodeling (include both outward and inward remodeling) is defined as the adaptation of vascular wall geometry in response to (patho)physiologic stimuli¹⁸. It includes a progressive and persistent change in vessel size and structural alterations of the vessel wall, including degradation and reorganization of the ECM. Vascular remodeling is a common feature both in atherosclerosis, after invasive vascular interventions, such as percutaneous transluminal coronary angioplasty, and in flow cessation- induced vessel retraction^{7, 8, 10, 18}. Pivotal in vascular remodeling are degradation of the elastic laminae and the ECM, vascular inflammation and SMC phenotypic changes. ApoE^{-/-} mice have been used to study vascular remodeling processes under pathophysiological conditions, and ligation of the carotid artery in these mice was employed to emulate flow cessation-induced vascular remodeling and inflammatory hyperplasia^{19,10,20}. In this model, remodeling processes are dependent on both macrophage content¹⁰ and macrophage-derived proteolytic activity²¹. The key role of the cystein protease catK in atherosclerosis⁵ and proteolysis of ECM and in particular collagen^{2, 22} led us to investigate the contribution of this protease in flow cessation-induced remodeling of the carotid artery. We here show that catK deficiency normalizes the excessive, inflammatory intimal hyperplasia observed in hyperlipidemic apoE^{-/-} mice, suggesting that catK is a key player in apoE^{-/-}-associated vascular remodeling in flow cessation. Moreover, we provide evidence that catK may do so by modulating apoE^{-/-} associated hyperinflammatory status rather than via its elastolytic activity.

First, we demonstrated that catK protein is more abundantly expressed in lesions of hyperlipidemic apoE^{-/-} compared to WT mice, and mainly co-localized with lesional leukocytes. In vivo studies revealed that intimal hyperplasia and vascular remodeling is much more pronounced in apoE^{-/-} than in WT mice. CatK deficiency was seen to only protect against macrophage-rich (apoE^{-/-}) but not macrophage-poor lesion formation (WT mice) and almost completely prevented the induced hyperplasia and remodeling observed in apoE^{-/-} mice. This suggests that catK is instrumental in the hyperlipidemia associated augmented hyperplastic response to flow cessation.

Consistent with previous reports^{10,20}, apoE^{-/-} mice show more pronounced expansive remodeling in response to flow cessation than WT mice. Interestingly, Eschert et al recently showed that apoE^{-/-} mice fed a normal chow diet displayed 'maladaptive'

constrictive remodeling, while cholesterol-fed apoE^{-/-} mice displayed outward/expansive remodeling²⁰. The extent of this phenomenon is overtly linked to macrophage content and function as intimal lesions of both WT and apoE^{-/-} mice fed a normal diet contained virtually no macrophages²⁰. Outward remodeling was postulated to be driven by macrophage expression of MMP, which was supported by the observation that MMP9^{-/-}/apoE^{-/-} mice showed a prominent reduction in the total vessel and intima area after carotid artery ligation²¹. In our study, catK deficiency induced a similar lesion phenotype and inhibited macrophage-rich lesion formation in the apoE^{-/-} mice. In contrast to the study by Lessner et al, in which MMP9 deficiency resulted in a decrease in collagen and SMC content, we did not observe any changes in the relative collagen and SMC content in lesions of catK deficient apoE^{-/-} and WT mice, despite a complete normalization of the aggravated remodeling response in the former. As discussed above, our study also did not reveal any effect of catK deficiency on elastin laminae fragmentation and macrophage proteolytic activity in both mouse strains. Apparently the reduced proteolytic activity in catK deficiency may only partly underlie the normalized vascular remodeling response to carotid artery ligation in apoE^{-/-} mice. Interestingly, in addition to its role in ECM degradation, catK was also reported to have proinflammatory activity by participating in inflammatory signaling pathways such as TGF- β^3 and TLR-9⁴. Therefore, we investigated the inflammatory status of catK deficient mice in more detail. Consistent with literature findings, apoE^{-/-} mice showed increased levels of circulating NK cells and decreased Treg cell numbers compared with WT mice. Of note, catK deficiency in apoE^{-/-} mice was accompanied by dramatically reduced systemic CD3⁺ and NK cell counts and an increase in Treg cell population, which in fact were normalized to base levels of WT mice, whereas no effects on these cell populations were seen in the WT mice.

CatK deficiency not only alter T-cell but also myeloid differentiation. Phenotypically, Ly6C^{high} subsets of monocytes are characterized as inflammatory monocytes. Ly6C^{high} monocytes have the capacity to migrate to sites of peripheral inflammation and are important for the resolution of inflammation, whereas Ly6C⁻ monocytes enter the tissues and replenish the tissue-resident macrophage and DC population²³. Surprisingly, apoE^{-/-} mice showed significantly decreased level of circulating CD11b⁺Ly6G⁻Ly6C^{high} monocytes compared with WT mice. This finding is not consistent with the previous report showing an elevated Ly6C^{high} monocyte in circulation in apoE^{-/-} vs WT mice²⁴. Interestingly, the blood monocyte population of catK^{-/-}/apoE^{-/-} mice was enriched in inflammatory CD11b⁺Ly6G⁻Ly6C^{high}, whereas that of resident Ly6C⁻ monocytes was significantly reduced compared with apoE^{-/-} mice. Thus, catK deficiency under apoE^{-/-} background normalized Ly6C^{high} monocyte population to base level of WT

background. The implications of this finding for the normalized vascular remodeling in catK deficiency remain to be addressed.

CatK deficiency may not only affect monocyte differentiation but also monocyte/macrophage polarization. Our data show that catK deficiency tends to shift non-stimulated apoE^{-/-} BMM to an anti-inflammatory M2a phenotype, which are deemed instrumental in wound healing responses^{25, 26}. CatK deficiency, however, had a more pronounced effect on LPS or IL-4 induced macrophage polarization towards M1 and M2, respectively. CatK deficiency in apoE^{-/-} mice led to an augmented polarization, whereas in WT BMM it favored M1 polarization. Combined, the inhibitory effects of catK deficiency on intimal hyperplasia in apoE^{-/-} mice is at most partly attributable to a shifted macrophage polarization.

As mentioned above, catK deficiency significantly reduced circulating CD3 T-cell numbers and increased the regulatory T cell subset in apoE^{-/-} but not WT mice. This raises the possibility that the observed immunomodulatory effects of catK deficiency are opportune in hyperlipidemic apoE^{-/-} mice only, attenuating the elevated systemic inflammatory level in apoE^{-/-} mice to that of normolipidemic WT mice. The fact that the increased hyperplastic and remodeling response of apoE^{-/-} mice was almost completely prevented in catK deficiency suggests that catK may directly or indirectly be responsible for the aggravated flow cessation associated vascular remodeling in apoE^{-/-} mice. Whether or not this is related to hyperlipidemia or to the absence of apoE remains to be determined. Interestingly subphysiological plasma levels of apoE were shown to inhibit intimal hyperplasia in an arterial injury model, suggesting that apoE may, independent of plasma cholesterol levels, inhibit intima formation²⁷. Thus, the systemic anti-inflammatory potential of catK deficiency in mice might lead to a protection against macrophage-rich lesion formation in the apoE^{-/-} mice, but not in the WT mice. The molecular mechanism underlying the anti-inflammatory potential of catK deficiency might involve TLR9 and TGF- β signaling pathway. CatK inhibition led to reduced TLR9 responses⁴ and stimulated TGF- β signaling³. Although TLR4 was reported to involve in outward arterial remodeling in apoE^{-/-} mice²⁸, the role of TLR9 in vascular remodeling is less defined. In this study, we focused on the role of catK deficiency on inflammatory status. Nevertheless, whether or not catK deficiency will influence lesion component of T cells, macrophage polarization or specifically TLR9 and TGF- β activity remains to be addressed.

In summary, catK deficiency completely normalized the increased vascular remodeling response to flow cessation in apoE^{-/-} mice, but did not affect macrophage-poor lesion formation in wild type mice. This identifies catK as a major culprit in flow cessation-induced inflammatory remodeling and possibly vessel retraction. Furthermore

our data suggest that catK deficiency does so by inhibiting the apoE^{-/-} associated hyperinflammatory status, rather than by attenuating ECM degradation.

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9

General Discussion

Inflammation and extracellular matrix (ECM) remodeling are common features of several vascular pathologies such as atherosclerosis, aortic aneurysm formation and neointima formation¹. In addition, inflammation and ECM degradation are linked at multiple levels during initiation and progression of these pathologies. Inflammatory cells such as monocytes/macrophages and T cells are ubiquitously involved at every phase of atherogenesis. In the early stage, monocyte-derived macrophages, smooth muscle cell (SMC) and T cells are involved in intimal thickening/intimal xanthoma formation^{2,3}. Cytokines, chemokines, growth factors and especially proteolytic enzymes secreted by the inflammatory cells such as macrophages and at a later stage also by T cells stimulate the degradation of the fibrous cap, which may lead to plaque rupture⁴. Another vascular disease that shows features of inflammation is an abdominal aortic aneurysm (AAA). Inflammatory cells such as macrophages, T cells, B cells and mast cells are present in AAA^{5,6}. In addition to inflammation, AAA is typified by a permanent dilation of the abdominal aorta as a result of matrix degradation by excessive protease activity⁷. During neointima formation, proliferation and migration of SMC from the media to the intima facilitated by ECM degradation, contribute to intima formation^{8,9}. In addition to SMC as the constituents of the neointima, inflammatory cells secrete proinflammatory cytokines and growth factors, which may facilitate their recruitment into the neointima^{10,11}.

Thus, in all three vascular pathologies, inflammation and matrix degradation are common denominators. In this thesis we explored the therapeutic potential of calcineurin-dependent immunosuppression (part 1). We hypothesized that calcineurin-NFAT signaling pathway inhibition protects against inflammation-related vascular pathology. In part 2, the potential role of cathepsin K (catK), a potent lysosomal cysteine protease, in these vascular pathologies were discussed. As ECM degradation by protease is a common feature of AAA and neointima formation, we hypothesized that deficiency of catK protects against these vascular pathologies. As inflammatory and proteolytic pathways are dependent, a link between the NFAT-dependent calcineurin and catK signaling pathways is discussed. The major findings of the experiments performed in this thesis are summarized in table 9.1. In short, we found that low-dose but not high-dose FK506 treatment protected against collar-induced atherosclerosis formation, whereas the low-dose was ineffective in preventing angiotensin II (Ang II)-induced AAA formation. Moreover, NFATC2 deficiency in hematopoietic cell lineages did deteriorate rather than protect against atherosclerosis formation. Finally, catK deficiency protected against ligation-induced neointima formation in apoE^{-/-} mice, leaving neointima formation in WT and aneurysm formation in apoE^{-/-} mice unaffected.

Table 9.1

<i>Treatment</i>		<i>Model</i>	<i>Outcome</i>	<i>Possible mechanism</i>
FK506	low-dose	Collar-induced atherosclerosis in apoE ^{-/-} mice	Reduced	Low and high dose of FK506 differentially skewed Th1/Th2 balance
	high-dose		No effect	
FK506 (low dose)		Ang II-induced AAA in apoE ^{-/-} mice	No effect	The distinct pathological characteristics of atherosclerosis and AAA formation might explain the differential effect
NFATC2 ^{-/-} BMT		Atherosclerosis in aortic arch in LDLr ^{-/-} mice	Enhanced	NFATC2-deficient T cells showed an enhanced proliferative activity and displayed a memory T cell phenotype more prone to migrate to the atherosclerotic plaque
CatK ^{-/-}		Ang II-induced AAA in apoE ^{-/-} mice	No effect	Compensatory increase in proteolytic activity exerted by catS/C in catK ^{-/-} /apoE ^{-/-} mice. Increase in circulating granulocytes and effector T-helper cells in catK ^{-/-} /apoE ^{-/-} mice.
CatK ^{-/-}		Ligation-induced neointima formation in apoE ^{-/-} mice	Reduced	CatK deficiency corrected apoE ^{-/-} associated proinflammatory effect on peripheral immune response whereas had no effect on peripheral immune response in C57Bl6 mice.
		Ligation-induced neointima formation in C57Bl6 mice	No effect	

Part 1: Immunosuppressive therapy in vascular disease

Local immunosuppressive effect of FK506 in atherosclerosis

FK506 is a widely used immunosuppressive drug that inhibits the calcineurin-NFAT signaling pathway. As shown in chapter 3, low- but not high-dose treatment with FK506 reduced collar-induced atherosclerotic lesion formation in apoE^{-/-} mice. We already discussed in chapter 3 that low- and high-dose FK506 affected atherogenesis differently probably by differentially skewing the Th1/Th2 balance. An alternative explanation could be that the presence of FK506-binding proteins (FKBPs) in atherosclerotic tissues is responsible for the dose- dependent effect of FK506 on atherosclerosis formation. Evidence suggests that the effects of FK506 are controlled by the relative expression profile of FKBP or immunophilins in particular tissues^{12, 13}. This implies that identical doses of FK506 could affect diverse tissues differently.

A large number of immunophilins belonging to the FKBP family have been discovered, with FKBP12 considered as the archetypal member¹⁴. It is generally recognized that

FK506 binds to FKBP12 and effectively inhibits the downstream calcineurin signaling pathway^{15, 16}. Interestingly, gene expression profiling of human in-stent-restenosis showed that FKBP12 was upregulated in the neointimal tissue retrieved by helix-cutter atherectomy¹⁷. Moreover, immunohistochemistry staining of FKBP12 revealed a significant increase in FKBP12 expression in human in-stent restenosis atherectomy specimens compared to that in primary stable atheroma¹⁸. This suggests that restenosis might be sensitive to FK506. Indeed, a FK506-eluting stent in both a porcine coronary artery model and a canine model, effectively suppressed neointima formation in comparison with bare stents^{19, 20}.

To investigate changes in FKBP expression profiles further, we have explored our existing microarray data base in which gene expression profiles of human atherosclerotic lesions are analyzed. Analysis showed that FKBP12 was significantly upregulated in stable advanced carotid lesions compared to early carotid lesions (fold change: 1.10255, $p=0.00032$). The fact that stable advanced atherosclerotic lesions had significantly higher expression of FKBP12 suggests that advanced atherosclerotic lesions compared to initial lesions might be more sensitive to FK506 treatment. Therefore, the low-dose of FK506 might exert its protective effect not only via a systemic immunosuppressive effect, but also at the level of the vessel wall by directly binding to FKBP12. Whereas at a high dose, FK506 might bind to other FKBP s (discussed in the next paragraph) and therefore might overrule the effect of FKBP12. The lack of sensitivity of AAA for FK506 may suggest that FKBP12 expression is low. However, we do not have data on FKBP12 expression in aneurysm tissue.

In addition, other FKBP family members such as FKBP38, 51, and 52 not only consist of a single FK506 binding domain (FKBD), but also contain additional functional units, such as tetratricopeptide repeat TPR domains, calmodulin binding and transmembrane motifs. Thus they were not only able to bind to FK506²¹, but are also associated with other functions²¹ such as heat shock protein 90-dependent signal transduction²², nuclear translocation of progesterone receptor, glucocorticoid receptor and ternary complexes²³. Thus, next to the level of expression of FKBP12 as a determinant of the local FK506 effect, it is possible that other FKBP s mediate actions beyond calcineurin inhibition. Theoretically, a high concentration of FK506 does not only bind to FKBP12, but also to other FKBP s (although the affinity to FK506 of these FKBP s is not as strong as FKBP12), which will mediate functions other than calcineurin inhibition. This might possibly explain the various side effects related to high dose FK506 treatment²⁴. In favor of this hypothesis, our microarray data showed that other FKBP s such as FKBP9, 10, 11, 14 were significantly enhanced in advanced atherosclerotic lesions compared to initial lesions. It indicates that these other FKBP s could 'overrule' FKBP12-FK506 interaction and even have less 'beneficial effects'.

Although a low-dose FK506 treatment reduced collar-induced atherosclerosis, low-dose FK506 treatment had no effect on Ang II-induced AAA formation in apoE^{-/-} mice (chapter 4). As discussed in chapter 4, the difference in the pathogenesis of atherosclerosis and AAA might explain this discrepancy. Atherosclerosis is an inflammatory Th1 mediated disease, whereas AAA is a permanent dilation of arterial wall and elastin degradation contribute critically to AAA formation⁷. Protease release by mast cells are thought to be involved in the pathogenesis of AAA⁶. Treating Ang II-infused apoE^{-/-} mice with a mast cell degranulation inhibitor significantly reduced the dilatation of the aorta and the inflammatory response compared with the untreated group²⁵. However, the effect of FK506 on mast cell protease release is not clear. Given the fact that a MMP inhibitor significantly inhibited AAA formation²⁶, it is possible that the inhibition of proteolytic activity is more effective than the inhibition of cytokine function in the treatment of AAA. Moreover, different from atherosclerosis, AAA formation was associated with increased TGF- β signaling and could be prevented by a TGF- β antagonist²⁷. FK506 on the other hand, activated TGF- β signaling in SMC²⁸ and mink epithelial cell line²⁹. This activation of TGF- β may have dominated over the immunosuppressive effect of low-dose of FK506 in AAA.

The lack of effect of FK506 on AAA could in addition be explained by the possible accumulation of FK506 in atherosclerotic lesions rather than in aneurysm lesions. In human transplantation recipients, FK506 blood concentrations are routinely monitored to ensure sufficient drug levels. However, the blood FK506 concentration is only a surrogate marker of that at the site of action^{30,31}. Ninety-five percent of FK506 binds to erythrocytes (bound fraction) and five percent resides in plasma (unbound fraction)³². The pharmacological activity is considered to be dependent on the unbound fraction of FK506. At steady state, FK506 is distributed extensively in the body and the majority of the drug resides in the tissues outside the blood compartment³². Thus, kidney accumulation of FK506 was found to be more than 30 times higher compared to 24-hour whole blood levels in mice, given a FK506 dose of 0.5 mg/kg/day²⁴.

To understand the pharmacokinetics of FK506, compound characteristics have to be considered. FK506 is highly lipophilic and might accumulate in atherosclerotic lesions in apoE^{-/-} mice. Given the high lipid content of atherosclerotic lesions in our model, in which mice are fed a western type diet, and the high lipophilic nature of FK506, local tissue dynamics of FK506 may be a factor in the sensitivity of the disease.

In our AAA study, however, AAA was induced by subcutaneous infusion of Ang II in apoE^{-/-} mice with normal diet. AAA lesions in apoE^{-/-} mice do not show abundant presence of foam cells and lipid core. As a consequence, there will be less FK506 accumulation in AAA compared to atherosclerotic lesions. To test this hypothesis, we have pursued the methods to evaluate tissue levels of FK506 in mice, but were unable

to optimize the method.

Translation of the FK506 dose from the mouse studies to clinical use and vice versa

FK506 dose appears as a critical factor in the potential therapeutic application of FK506 in atherosclerosis. However, an appropriate method to translate FK506 dose from mouse study to clinical practice is lacking. The Food and Drug Administration has suggested to use the normalized body surface area (BSA) to translate the dose used in animals studies to the starting dose in clinical trial, which is represented in mg/m^2 ³³. The human equivalent dose (HED) to be converted from the dose used in mouse, can be calculated using the formula shown in reference³³. To convert the low-dose FK506 that we used in our mouse studies to the HED, 0.05 mg/kg (FK506 low-dose in mouse) is multiplied by the Km factor (3) for a mouse³³ and then divided by the Km factor (37) for a human³³ (with Km representing body weight to BSA ratio). This calculation resulted in a HED of 0.004 mg/kg. With this method, we can also translate the clinically used FK506 dose to mouse. A dose between 0.05 mg/kg and 0.25 mg/kg of FK506 is used clinically in human, and this is equivalent to a dose in the range between 0.62 mg/kg and 3.08 mg/kg for mouse. Therefore, the high dose FK506 treatment of 1 mg/kg/day in mice in our study is within the dose range in transplanted patients. Furthermore, if we want to translate the low-dose regime from mouse study to clinical use, a FK506 concentration of 0.004 mg/kg might be a starting point.

VIVIT treatment-an alternative way to study the role of NFAT in vascular pathologies?

As mentioned previously, we found that low- but not high-dose of FK506 reduced collar-induced atherosclerosis formation in apoE^{-/-} mice¹³. FK506 is a widely used immunosuppressive drug that inhibits the calcineurin-NFAT signaling pathway.

Calcineurin, a phosphatase, controls the function of miscellaneous effector proteins, ranging from transcription factors to enzymes, transmembrane ion channels, and proteins involved in apoptosis³⁴. The major setback of calcineurin inhibitors (FK506/Cyclosporine A) is that they intervene in calcineurin activity, and not only dephosphorylate NFAT, but also affect other downstream signal transduction pathways of calcineurin (such as protein kinase C activation or calcineurin-mediated NF- κ B activity), resulting in undesired side effects and toxicity such as nephrotoxicity, and hypertension³⁵. Several mechanisms have been postulated to explain the nephrotoxicity and hypertension due to calcineurin inhibition³⁶. Firstly, calcineurin inhibition induces endoplasmic reticulum stress, which in turn regulates activities of pro-apoptotic BCL-2 family protein BCL-2-associated agonist of cell death (BAD)³⁷. This may induce apoptosis and renal toxicity³⁸. Secondly, hypertension was reported as an important side effect of

immunosuppressive therapy. Thus calcineurin inhibition, lead to reduced prostacyclin and nitric oxide synthesis and enhanced thromboxane and endothelin release, leading to vascular constriction^{39, 40}. Furthermore, FK506 and cyclosporine A have been shown to be associated with an increase in allograft vasculopathy, suggestive of a hampered tolerogenic response⁴¹. In our in vitro study in chapter 3, both low- and high-dose of FK506 in vitro treatment on Jurkat T cells significantly reduced nuclear expression of NFATC2, whereas there was no difference in NFATC2 expression between the low- and high-dose treatments. The dose-dependent effect of FK506 on collar-induced atherosclerosis formation might result from unwanted calcineurin inhibition with high dose treatment. In order to circumvent inhibiting unwanted targets in calcineurin signaling pathway such as BAD³⁷, we choose to focus on one component of the signaling pathway NFAT.

NFATC1, C2 and C3 are the three NFAT family members that are expressed in the immune system and involved in calcineurin signaling pathway. During calcineurin inhibition, the translocation of NFAT proteins from the cytoplasm to the nucleus of activated cells is suppressed⁴². Indeed, studies by both our group and others showed that FK506 treatment suppressed NFATC1, NFATC2 and NFATC3 protein expression in the nucleus of activated T cells (chapter 3)⁴³. Suppression of NFATC1, C2 and C3 activity might therefore explain the athero-protective effect of low-dose FK506 treatment. However, we chose to study the effect of NFATC2 deficiency instead of C1 and C3 on atherosclerosis formation (reasons see below, chapter 5). We hypothesized that leukocyte NFATC2 deficiency protects against atherosclerosis for the following reasons. Firstly, intervention in the calcineurin-NFAT pathway by low-dose FK506 was earlier shown to be atheroprotective¹³, although the actual molecular NFAT target mediating this effect remains to be identified. Secondly, NFATC2 was recently reported to control regulatory T-cell function and its deficiency led to a Th2 biased T-cell response⁴⁴⁻⁴⁶, both processes that were shown to attenuate the atherogenic response. Thirdly, NFATC2 deficient mice display an enhanced allergic phenotype characterized by eosinophilia, enhanced IgE production and increased production of Th2 cytokines in vivo and in vitro^{45, 47-49}. Finally, both NFATC1 and C3 deficient mice appeared to be involved in processes that might aggravate atherosclerosis. Thus NFATC1-deficient T cells showed impaired production of IL-4 and other Th2 cytokines and reduced titres of IgE^{50, 51}, and NFATC3 deficiency resulted in a mild hyperactivation of peripheral T cells⁵².

Contrary to what we expected, leukocyte NFATC2 deficiency deteriorated atherosclerosis. We found that NFATC2^{-/-} bone marrow transplanted mice displayed an enhanced rather than compromised immune response. Thus, NFATC2 deficient T cells had enhanced proliferative activity, displayed memory/activated T cell phenotype, and had robust migration capacity to atherosclerotic lesions. Our FACS data further

showed a reduction in CD4⁺/CD8⁺ ratio and enhanced Fox3⁺ regulatory T cells (Treg) in NFATC2 deficient chimeras, however the functional consequence of these circulating immune response for plaque development needs further validation.

The enhanced immunological responses in the NFATC2^{-/-} chimera might disqualify NFATC2 as a proper target to prevent atherosclerosis development. However, the enhanced immunological responses in NFATC2^{-/-} chimeras could be an unwanted consequence of redundancy in NFAT-regulated function. It is possible that other NFAT family members or cofactors have compensated for the lack of NFATC2 in maintaining an immunocompetent status in NFATC2 deficiency^{47, 53}. Indeed western blot analysis of whole cell lysates showed increased levels of NFATC1 and NFATC3 in NFATC2^{-/-} CD4⁺ T cells⁵⁴. The high degree of similarity of the Rel-homology regions among the different NFAT family members indicates common DNA-binding specificities, which might further explain the redundancy in some NFAT-regulated functions⁵⁵. Thus the observed enhanced immune response in NFATC2^{-/-} chimeras might be caused by a compensatory activation of other NFAT members. NFATC2 knockout mice therefore might not represent the ideal tool to study the function of NFATC2. Targeted NFATC2 inhibition, due to its function in Th2 mediated response could still be a promising target in the treatment of atherosclerosis. However a rather 'smart' therapy that specifically intervenes in NFATC2 without activating other NFAT members or of NFAT independent pathways is needed. VIVIT-an alternative inhibitor of calcineurin-NFAT signaling pathway could be a choice. Being a promising inhibitor of calcineurin-NFAT signaling, VIVIT is a small organic molecule inhibitor of the calcineurin-NFAT signaling pathway, which specifically inhibits binding of the calcineurin to its substrate NFAT and acts at the protein-protein contact and not at the calcineurin catalytic site³⁴. VIVIT displays a preference for NFATC2 compared to other isoforms⁵⁶. VIVIT, unlike FK506 and cyclosporine A, displays a favourable therapeutic profile as it does not inhibit calcineurin signaling, which could initiate unwanted toxic effects. VIVIT has been tested in several cardiovascular diseases and proved to prevent cardiac myocyte hypertrophy in animal models and reduce neointima formation and neointimal SMC proliferation in a rat carotid artery balloon injury model (reviewed in ⁵⁶).

However, the effect of VIVIT on atherosclerosis has not been investigated. Although VIVIT could efficiently hinder calcium/calmodulin activated NFATC1, NFATC2, and NFATC3 dephosphorylation, it displays a clear preference for NFATC2 compared to other isoforms⁵⁶. Therefore, as an effective inhibitor of NFATC2 signaling pathway, VIVIT treatment might be considered an alternative to study the role of NFATC2 in atherosclerosis. Unlike the possible compensatory activation of other NFATs in the NFATC2^{-/-} mice, VIVIT treatment is unlikely to induce a compensatory activation of other NFAT isoforms⁵⁶.

Part 2: CatK- friend or foe in vascular pathologies?

CatK is a potent lysosomal cysteine protease involved in ECM degradation and inflammatory responses. In an earlier study from our laboratory, systemic disruption of the catK gene reduced atherosclerosis lesion growth, but also enhanced macrophage foam cell formation by increased lipid uptake through CD36 and caveolins^{57, 58}. Guo et al demonstrated that the absence of leukocyte catK in BMT recipient mice decreased collagen and increased macrophage content of atherosclerotic lesions while lesion size was not affected⁵⁹. These findings indicate that abolishing catK in leukocytes is detrimental for plaque stability.

In chapter 7, we studied the effect of systemic catK deficiency on AAA formation. Unexpectedly, we showed that catK deficiency in apoE^{-/-} mice did not confer protection against AAA formation, and did not affect the number of elastin breaks and or proteolytic activity in AAA lysates. In contrast, our previous study demonstrated that the number of elastin breaks in the media underlying the atherosclerotic plaque even was substantially decreased in advanced lesions of catK^{-/-}/apoE^{-/-} compared with apoE^{-/-} mice⁵⁷. The differential outcome of catK deficiency in these two vascular pathologies indicated fundamental differences in the proteolytic and possibly inflammatory processes that underlie AAA and atherosclerosis. In Ang II- induced AAA lesions, catS and catC-positive cell numbers were significantly increased in both the aneurysm and adventitia of catK^{-/-}/apoE^{-/-} mice compared with apoE^{-/-} mice, which might have compensated for the deficiency of catK-derived proteolysis in the aneurysm tissue of catK deficient apoE^{-/-} mice. However, catS and catC protein expression was not significantly different in atherosclerotic lesions of catK^{-/-}/apoE^{-/-} compared with apoE^{-/-} mice⁵⁷. This discrepancy could be explained by the possible effect of the Ang II infusion needed to induce AAA. There was a significant increase in circulating granulocytes and activated T cells numbers in catK^{-/-}/apoE^{-/-} mice with Ang II infusion but not in control mice. Indeed, Ang II was reported to contribute to several steps in the inflammatory cascade that appear during AAA⁶⁰. Although our in vitro study on BMM showed that Ang II treatment by itself could not significantly enhance catS and catC mRNA expression, this may not reflect the in vivo situation in pathological lesions, where catS and catC-positive cells were more abundantly present in aneurysm lesions. To avoid the unwanted pro-inflammatory effect of Ang II, intraluminal infusion of elastase and periaortic incubations of calcium chloride might be a better model to study the effect of catK deficiency in AAA formation⁷.

Proteolytic arterial remodeling and inflammatory responses are present in vascular remodeling such as AAA formation⁶ and intima formation. In chapter 8, we studied

the role of catK deficiency on vascular remodeling and neointima formation in a carotid artery flow cessation model in catK-deficient apoE^{-/-} and wt mice. We found that catK deficiency in the apoE^{-/-} mice significantly protected against macrophage-rich neointima formation, but not against macrophage-poor neointima formation in wt mice. Furthermore, we discussed the theory that catK deficiency reversed the aggravated remodeling response in hyperlipidemic apoE^{-/-} mice, suggesting that catK could directly or indirectly be a critical player in flow cessation-induced vascular remodeling. This profound effect was attributed not so much to catKs' capacity to degrade ECM, but rather to its anti-inflammatory activity on peripheral immune activity. In the next paragraph, we will therefore focus on the potential anti-inflammatory effects of catK deficiency.

As macrophage-rich lesion formation is associated with increased macrophage foam cell content¹⁰ and various studies showed that macrophage-derived inflammatory growth factors contribute to intima lesion enlargement^{11, 61}, we studied basal and cytokine-induced polarization of BMM. Surprisingly, catK deficiency in apoE^{-/-} background skewed macrophages towards M2a phenotype. Intriguingly, after in vitro stimulation with LPS or IL-4, catK^{-/-}/apoE^{-/-} macrophages were tended to be polarized to either M1 or M2 phenotype, which is still hard to explain. Our earlier study showed that catK deficiency reduced plaque progression but enhanced macrophage foam cell formation⁵⁷ possibly through increased lipid uptake mediated by CD36 and caveolins⁵⁸, indicating a less favorable role of catK deficient macrophages in protecting against atherosclerosis formation. Combined, the inhibitory effect of catK deficiency on intimal hyperplasia in apoE^{-/-} mice is at most partly attributable to a shifted macrophage polarization to M2a phenotype.

We further investigated the peripheral inflammatory status of catK deficient mice in more detail using FACS analysis. Interestingly, catK deficiency in apoE^{-/-} mice was accompanied by a reduction in systemic CD3 cell populations and an enhanced Treg cell population, whereas no effects on these cell populations were seen in the WT mice. This raises the intriguing hypothesis that the observed immunomodulatory effects of catK deficiency are opportune only in hyperlipidemic apoE^{-/-} mice, which are typified by an increased systemic inflammatory status compared to WT mice. However, the molecular mechanism underlying the anti-inflammatory effect of catK deficiency is not clear. To our knowledge, this is the first report showing that catK deficiency reduced circulating CD3 cell and enhanced Treg population in apoE^{-/-} mice. The enhanced Treg numbers in catK^{-/-}/apoE^{-/-} mice might involve toll like receptor 9 (TLR9) signaling, as a role for TLR9 signaling in the regulation of Treg function has been reported. Stimulation of Treg with TLR9 agonists, resulted in decreased IL-10 and IFN- γ synthesis and a concurrent loss of regulatory function in Treg⁶². TLR9 responses are recently reported

to be involved in catK signaling^{63, 64, 65}, in that catK inhibition led to compromised TLR9 responses^{63, 64}. Administration of the potent orally active catK inhibitor, NC-2300, in rats with adjuvant-induced arthritis not only suppressed bone resorption by osteoclasts, but also instigated anti-inflammatory effects, resulting in reduced paw swelling. Further studies showed that catK inactivation led to the blockade of essentially all the downstream pathways of TLR9 signaling in dendritic cells, showing a crucial role for catK in TLR9 signaling⁶³. The possible mechanism for the involvement of catK in TLR9 signaling was further studied by Ewald et al⁶⁵. It was shown that the cleaved form of TLR9 in endo-lysosomal pathway was able to recognize and bind to CpG ligand and recruit MyD88 on activation, whereas full-length TLR9 lost this recruiting ability. It indicated that the truncated receptor, rather than the full-length form, is functional. Furthermore, blocking TLR9 cleavage by inhibiting various cellular proteases resulted in a non-functional receptor⁶⁵. Thus inhibition of catK using protease inhibitors prevented TLR9 signaling function^{63, 64}. However in a study using transgenic mice, macrophages and dendritic cells from catK^{-/-}, catS^{-/-}, catB^{-/-}, and catS/catL^{-/-} mice did not show defective TLR9 signaling⁶⁵. This study suggest that multiple proteases instead of single cathepsin are needed for functional processing of the receptor⁶⁵.

In our study, catK deficiency in apoE^{-/-} background might lead to a compromised TLR9 response which reduces Th1 response, limits inflammatory reaction and in addition might deactivate plasmacytoid dendritic cell (pDC)⁶⁶, as a consequence reduces macrophage-rich lesion formation. However, the function of pDC in vascular remodeling is unknown. Whether or not catK deficiency resulted in reduced Th1 response needs further study.

Part 3: CatK is regulated by NFATC1

Inflammatory and proteolytic pathways at times act in concert in the development of vascular diseases. Infiltrating leukocytes are not only a prominent feature of atherosclerotic and AAA lesions, they also functionally contribute to lesion progression due to the release of cytokines, chemokines and growth factors. In addition, different subsets of leukocytes regulate vascular remodeling by producing proteases. In this thesis, we focused on the calcineurin pathway in view of the therapeutic possibility of immunosuppressive therapy in several vascular pathologies. However, studies in literature describe a link between NFATC1 as a component of calcineurin signaling pathway and catK.

At the molecular level, NFAT and especially NFATC1 was found to stimulate catK expression through Receptor Activator of NFκB ligand (RANKL)-associated signaling in certain cell types (reviewed in ⁶⁷). RANKL via the cytoplasmic membrane receptor RANK

(receptor activator of NF κ B), induced both osteoclast differentiation and activation⁶⁸. RANKL was able to stimulate catK mRNA expression in isolated mature rat osteoclasts⁶⁹. Moreover, RANKL was reported to be present in the ECM surrounding the calcium mineral deposits of the plaques⁷⁰. In addition, RANKL leads to the phosphorylation of NFATC1 by p38 mitogen-activated protein kinases, thereby inducing translocation of NFATC1 into the nucleus and binds to the *cis*-elements, N1 and N2, that are present in the upstream promoter region of the catK gene⁷¹. Cyclosporine A, an inhibitor of the phosphatase activity of calcineurin, inhibited NFAT activation and repressed RANKL-induced catK mRNA expression⁶⁷. Recently, NFATC1 binding sites were identified on the transcription initiation site of the catK promoter⁷². In conclusion, the fact that NFATC1 signaling is a functional regulator of catK, illustrates the interaction between inflammatory and proteolytic pathways. However whether or not FK506 treatment or NFATC2 deficiency inhibited catK activity and possibly catK related proteolytic activity was not reported and need further study.

Concluding remarks

Although inflammatory processes and vascular remodeling are common features of several vascular pathologies such as atherosclerosis and aneurysm formation, calcineurin targeted immunosuppressive therapy only protected against atherosclerosis formation, but was completely ineffective in aneurysm formation. Likewise, catK deficiency protected against macrophage-rich neointima formation leaving macrophage-poor neointima and aneurysm formation unaffected, suggesting that although inflammatory and proteolytic pathways contribute to these vascular pathologies, immunosuppressive or anti-proteolytic therapy was not equally effective.

The differences in the underlying inflammatory pathophysiology (discussed in chapter 4) and possibly different stimulus to induce mouse model of atherosclerosis and AAA formation might explain the different outcome after low dose FK506 treatment. The more enhanced inflammatory status in apoE^{-/-} mice could explain our observation that catK deficiency only protected against macrophage-rich, but not macrophage-poor neointima formation. It suggested that the anti-inflammatory effect of catK deficiency was evident only on a hyperlipidemic and hyperinflammatory (apoE^{-/-}) background. While immunosuppressive agents are widely used in clinical practice to improve survival of transplanted organs, several side effects and a toxic profile limit their use. Although application of a lower dose for FK506 or cyclosporine A could be an option, this will compromise the required immunosuppressive effect. Further studies on the signaling pathways would enable to identify the major cellular 'actor' that is responsible for the therapeutic effect. In this way, a small peptide VIVIT was developed that intervened in NFATC2 signaling pathway specifically without inhibiting calcineurin function and

without inducing a compensatory activation of other NFAT members. Furthermore, abundant FKBP12 expression was found in intimal lesions and already proved to be a novel target for intima development. This could lead to the design of selective ligand that specifically binds to FKBP12.

Interventions in proteolytic pathways are also promising for osteoporosis and probably atherosclerosis. Relacatib is a catK inhibitor that has been validated in the treatment of osteoporosis and proved effective⁷³. Furthermore, several catK inhibiting compounds are being tested in clinical trials to study their effects on osteoporosis or osteoarthritis, and efficient inhibition of bone resorption and improved bone formation has been shown^{74, 75}. However, our study shows that the mode of action for catK is not solely confined to proteolysis but also affects inflammatory processes. The recent discovery of the involvement of catK in atherosclerosis and neointima formation warrants further investigation of the therapeutic potential of catK inhibitors in these vascular pathologies. Nevertheless, adverse effect of catK inhibition on macrophage function should be avoided. The most promising therapy should specifically target the key component of the signaling pathway that contributes to disease progression without the potential side effects.

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Summary

Inflammation and extracellular matrix (ECM) remodeling are common features of several vascular diseases such as atherosclerosis, aortic aneurysm formation and vessel remodeling. Inflammatory cells are present in various stages of vascular diseases and can secrete proteolytic enzymes that contribute to ECM degradation and therefore vascular remodeling. This suggests that inflammation and ECM degradation act not in an independent manner but in mutual interaction and are linked at multiple levels in these vascular diseases. In this thesis, we investigated two hypotheses on the therapeutic potential of immunosuppressive and anti-proteolytic therapy in several vascular diseases. Firstly, as the calcineurin-NFAT signaling pathway was seen to be essential for an adequate immune response, we argued that inhibition of this pathway will protect against the aforementioned vascular diseases. Secondly, proteolytic arterial remodelling is a key process in aneurysm formation, atherosclerosis and neointima formation, therefore we hypothesized that inhibition of a key protease, cathepsin K, will protect against several vascular diseases that are characterized by excessive ECM remodeling.

Chapter 2 provides an update of current literature on the potential of immunomodulatory drugs as therapeutic modality for cardiovascular disease. After a short overview of the specific inflammatory pathways involved in atherosclerosis, we have reviewed the perspectives of several immunosuppressive drugs that are currently used for the prevention of (organ) transplant rejection (e.g. FK506, sirolimus, cyclosporine A and mycophenolate mofetil), for treatment of (clinical) atherosclerosis.

FK506 is a widely used immunosuppressive drug that inhibits the calcineurin-NFAT signaling pathway. Whereas we have previously shown that low-dose treatment with FK506 attenuated the progression of murine atherosclerosis, other studies in cholesterol-fed rabbits reported that high dose FK506 treatment deteriorated atherosclerosis. In **chapter 3**, we therefore hypothesized that the dosage of FK506 is critical for its effects in the initiation of atherosclerosis. This led us to investigate dose-dependent effects of FK506 in our mouse model of collar-induced atherosclerosis. Unlike low-dose FK506, high-dose FK506 did not protect against atherosclerosis. We provide evidence for a dose dependent effect of FK506 on peripheral immune activity. Cytokine analysis of conditioned media of CD4⁺ T cells isolated from FK506-treated mice revealed that low-

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dose FK506 significantly increased the IL-4/IFN- γ and IL-10/IFN- γ ratio suggestive of moderate Th2 skewing, whereas high-dose treatment decreased this ratio. These results unveil dosage as a critical factor in the effects of FK506 on atherosclerosis, probably by differentially skewing the Th1/Th2 balance.

An aneurysm is a permanent pathological dilation of the arterial wall, and is prone to rupture. Inflammatory processes play a significant role in abdominal aortic aneurysm (AAA) formation. Given the inflammatory features of AAA and the potential immunosuppressive effect of low-dose FK506, we hypothesized that low-dose FK506 treatment could decrease AAA formation. In **chapter 4**, we describe the effect of low-dose FK506 treatment on angiotensin II (Ang II)-induced aneurysm formation in the abdominal aorta of apoE^{-/-} mice. Contrary to our expectations, FK506 treatment did not protect against murine AAA formation. The distinct underlying pathophysiology of atherosclerosis and aneurysm formation might explain the differential effect of low-dose FK506 in these vascular pathologies.

As intervention in the calcineurin-NFAT pathway by low-dose FK506 treatment was shown to be atheroprotective, we further investigated the effect of a deficiency of one of the potential FK506 targets, NFATC2, in the hematopoietic lineage on atherosclerosis in **chapter 5**. Surprisingly, NFATC2 deficiency in haematopoietic cells resulted in more advanced lesion development and CD3⁺ T cell content was sharply increased in NFATC2^{-/-} plaques. We found that NFATC2 deficiency result in an enhanced rather than compromised immune response in that NFATC2 deficient T cells had enhanced proliferative activity, displayed a memory/activated T cell phenotype, and had robust migration capacity to atherosclerotic lesions. These findings suggest that NFATC2 deficiency enhances immune responses critical for atherosclerosis progression.

Chapter 6 presents a literature review on the role of cathepsins in vascular remodeling and atherosclerosis.

In **chapter 7**, we focused on the effect of lysosomal cysteine protease-cathepsin K (catK) in AAA. CatK exerts strong elastinolytic and collagenolytic activity and is implicated in a wide range of pathological disorders including cardiovascular diseases. CatK expression was found to be elevated in human aortic aneurysm pointing to a role in this vascular disease. Conceivably a deficiency of catK could decrease aneurysm formation. To test this hypothesis, we investigated the effect of catK deficiency on Ang II-induced aneurysm formation in the abdominal aorta of apoE^{-/-} mice. Much to our surprise, catK deficiency did not protect against murine aneurysm formation, nor did it affect medial

elastin breaks. Proteolytic activity in abdominal aortic lysates was comparable between apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice. Adventitial presence of catS and catC-expressing cells was significantly increased in catK^{-/-}/apoE^{-/-} versus apoE^{-/-} mice, which might have compensated for the deficiency of catK-derived proteolysis in the aneurysm tissue of catK deficient apoE^{-/-} mice. Moreover, circulating granulocyte and activated T cell numbers were significantly increased in Ang II-infused catK^{-/-}/apoE^{-/-} mice compared to apoE^{-/-} mice. In conclusion, the finding that catK deficiency did not protect against aneurysm formation is remarkable given the previously reported unfavorable effects of this protease in atherosclerosis and once again underpins the fundamental differences in the proteolytic processes that underlie AAA and atherosclerosis.

This finding has prompted to a study of the role of catK in another vessel wall pathology, flow cessation-elicited carotid artery remodeling, under conditions of normo- (wild type/WT mice) and of hyperlipidemia (apoE^{-/-} mice) (**chapter 8**). Surprisingly, catK deficiency completely blunted the augmented hyperplastic response to flow cessation in apoE^{-/-} (macrophage-rich lesions), while having no effect at all on vascular remodelling in WT mice. Interestingly, catK deficiency significantly reduced circulating CD3 T-cell numbers, but increased the regulatory T cell subset in apoE^{-/-} but not WT mice. In addition, apoE^{-/-} hyperplastic remodeling was characterized by significantly enhanced number of circulating natural killer cells and reduced numbers of regulatory T cells in spleen compared to WT mice. Furthermore, catK deficiency tended to bias macrophage polarization towards an M2a phenotype, which is instrumental in wound healing responses. Therefore, catK deficiency almost completely blunted the increased vascular remodeling response of apoE^{-/-} mice to flow cessation, possibly by correcting hyperlipidemia-associated pro-inflammatory effects on peripheral immune response.

In **chapter 9**, the findings and implications of the results presented in this thesis are discussed in relation to the available literature.

Inflammatory processes and vascular remodeling are common features of several vascular pathologies such as atherosclerosis and aneurysm formation. Nevertheless, an immunosuppressive therapy only protected against atherosclerosis formation and was completely ineffective in aneurysm formation. Likewise, catK deficiency protected against macrophage-rich lesion formation but not macrophage-poor lesion and aneurysm formation, suggesting that although inflammatory and proteolytic pathways contribute to all of the aforementioned pathologies, immunosuppressive or anti-proteolytic therapy are not equally effective. While immunosuppressive agents are widely used in clinical practice to improve survival of transplanted organs, several side effects

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and a toxic profile that is related to calcineurin inhibition, limit their use. Alternative solutions for immunosuppressive therapy in the treatment of vascular pathologies include refinement/optimization of the dosage, disease-specific interventions and novel small-peptide inhibitors that more specifically interfere with the calcineurin/NFAT pathway. Interventions in proteolytic pathways are also promising for the treatment of atherosclerosis. The recent discovery of the involvement of catK in atherosclerosis and neointima formation warrants further investigation of the therapeutic potential of catK inhibitors in these vascular pathologies. The most promising therapy should specifically target the key component of the signaling pathway that contributes to disease progression without the potential side effects.

Nederlandse samenvatting

(Dutch summary)

Verschillende aandoeningen van de grote vaten zoals aderverkalking (atherosclerose), verwijding van de grote buikslagader (aneurysma vorming) en structuurveranderingen van de vaatwand (vaatwand-remodelering) tijdens neointima vorming, worden gekenmerkt door ontstekingsprocessen en veranderingen in het bindweefsel, ook wel de extracellulaire matrix (ECM) genoemd. Deze ontstekingsprocessen en veranderingen in de ECM zijn bovendien onderling aan elkaar gerelateerd, aangezien ontstekingscellen enzymen (proteases) kunnen uitscheiden die de ECM kunnen afbreken. In dit proefschrift werden 2 hypothesen onderzocht. De eerste hypothese richtte zich op het afremmen van de ontstekingsreactie. Calcineurine en NFAT zijn belangrijke moleculen in de ontstekingsreactie. Onze veronderstelling was dat remming van deze moleculen beschermend zou werken tegen atherosclerose en aneurysma vorming. De tweede onderzoeksvraag betrof een belangrijke protease, cathepsine K, die betrokken zou zijn bij verschillende aandoeningen van de grote vaten. Onze hypothese was dat remming van cathepsine K zou beschermen tegen vaataandoeningen die gepaard gaan met veranderingen in de ECM, zoals aneurysma en vaatwand-remodelering.

In **hoofdstuk 2** wordt een overzicht gegeven van de huidige literatuur over het gebruik van ontstekingsremmers bij de bestrijding van vaataandoeningen. Deze immunosuppressiva, zoals FK506, sirolimus en cyclosporine A worden nu met name gebruikt om afstoting tegen te gaan na orgaantransplantatie.

FK506 is een veelgebruikt immunosuppressivum dat aangrijpt op de eerder genoemde moleculen calcineurine en NFAT. Hoewel we eerder hebben aangetoond dat een lage dosis FK506 de progressie van atherosclerose in muizen verlaagde, liet een studie in konijnen zien dat FK506 de ontwikkeling van atherosclerose juist verergerde. In deze konijnenstudie werd echter een relatief hoge dosis FK506 toegediend. In **hoofdstuk 3** hebben we onderzocht in hoeverre de dosis een kritische factor is, in het effect van FK506 op de ontwikkeling van atherosclerose. In een muis model van atherosclerose (apoE deficiënte muizen) zijn de dosisafhankelijke effecten van FK506 vervolgens bestudeerd. In tegenstelling tot een lage dosis FK506, beschermde een hoge dosis FK506 niet tegen de ontwikkeling van atherosclerose. Verdere analyse in celkweken, met geïsoleerde (CD4-positieve) T cellen uit FK506 behandelde muizen, toonde aan dat een

lage dosis FK506 secretie van bepaalde ontstekingsmediatoren (cytokines) die wijzen op een ontstekingsremmende werking (Th2 respons) significant verhoogde, terwijl een hoge dosis FK506 juist zorgde voor ontstekingsbevorderend effect (Th1 respons). Deze resultaten tonen aan dat de dosering een kritische factor is bij de effecten van FK506 behandeling van atherosclerose, en dat de Th1/Th2 balans hier mogelijk een belangrijke rol in speelt.

Een aorta aneurysma is een verwijding van de vaatwand van een slagader. Het is aangetoond dat ontstekingsprocessen een cruciale rol spelen in de ontwikkeling van een aneurysma van de grote buikslagader, ook wel een abdominaal aorta aneurysma (AAA) genoemd. Gezien het feit dat ontsteking een belangrijke rol speelt in de ontwikkeling van AAA en dat een lage dosis FK506 de ontstekingsactiviteit onderdrukt, stelden we de hypothese dat een lage dosis FK506 de ontwikkeling van een AAA voorkomt. In **hoofdstuk 4** wordt het effect van een lage dosis FK506 beschreven in de ontwikkeling van aneurysma's in een muismodel. In dit model ontstaat door een continue infusie van het molecuul angiotensine II (Ang II) een aneurysma in de abdominale aorta van apoE deficiënte muizen. In tegenstelling tot onze verwachtingen beschermde FK506 niet tegen AAA vorming in deze muizen. De aard van de ontstekingsmechanismen van atherosclerose en aneurysma zijn blijkbaar verschillend, met als gevolg dat FK506 geen effect heeft op de ontwikkeling van aneurysma's in ons muismodel.

Zoals eerder vermeld in hoofdstuk 3, verminderde een lage dosis FK506 de ontwikkeling van atherosclerose. In **hoofdstuk 5** hebben we in meer detail onderzocht of de beschermende werking van FK506 terug te voeren is op een remming van NFATc2, één van de aangrijpingspunten van dit immunosuppressivum. Hierbij werd gebruik gemaakt van NFATC2 deficiënte muizen; de beenmergcellen van deze muizen zijn daarbij getransplanteerd in atherosclerotische muizen, waarna de effecten op het ontstaan van atherosclerose zijn bestudeerd. Onze hypothese was dat de afwezigheid van NFATC2 in, van het beenmerg afkomstige ontstekingscellen, zou beschermen tegen het ontstaan van atherosclerose. In tegenstelling tot onze verwachtingen, leidde NFATC2 deficiëntie tot ontwikkeling van grote gecompliceerde atherosclerotische lesies, met een verhoogd aantal (CD3-positieve) T cellen. Bovendien leidde NFATC2 deficiëntie tot een verstoorde algehele ontstekingsactiviteit. NFATC2 deficiënte T cellen waren actiever en vertoonden zowel een verhoogde celdeling als een verhoogde bewegingsactiviteit (verhoogde T cel migratie) richting de atherosclerotische lesie. Deze bevindingen suggereren dat NFATC2 deficiëntie bijdraagt aan de verdere progressie van atherosclerose door een verhoging van de ontstekingsactiviteit.

In **hoofdstuk 6** wordt een overzicht gegeven van de huidige literatuur over de rol van cathepsines in het herstructureren van de vaatwand (vasculaire remodelering) en atherosclerose.

In **hoofdstuk 7** bestuderen we het effect de protease cathepsin K in AAA. Cathepsine K kan de ECM componenten elastine en collageen zeer efficiënt afbreken. Onderzoek heeft aangetoond dat cathepsine K verhoogd aanwezig is in humane AAA, wat suggereert dat cathepsine K een rol speelt in deze aandoening. Onze hypothese was dat afwezigheid van cathepsine K, aneurysma vorming zal verminderen. Om dit te testen werd het in hoofdstuk 4 beschreven model gebruikt, waarin met behulp van een continue Ang II infusie een aneurysma van de buikslagader is opgewekt in muizen met een deficiëntie in cathepsine K en apoE. In tegenstelling tot onze verwachtingen, beschermde cathepsine K deficiëntie niet tegen aneurysma vorming in deze muizen. Nader onderzoek van het aneurysma weefsel toonde aan dat het vermogen om ECM componenten af te breken vergelijkbaar was in cathepsine K deficiënte en controle muizen. Dit werd waarschijnlijk veroorzaakt door de verhoogde aanwezigheid van 2 vergelijkbare cathepsines: er werden namelijk meer cathepsine S- en C-positieve cellen gevonden in het aneurysma weefsel van de cathepsine K deficiënte muizen. Bovendien was de algehele ontstekingsactiviteit in de cathepsine K deficiënte muizen duidelijk verhoogd na Ang II infusie (meer granulocyten en geactiveerde T-cellen aanwezig in de bloedbaan). Concluderend kan gesteld worden dat afwezigheid van cathepsine K wel de ontwikkeling van atherosclerose kan remmen, maar geen effect heeft op aneurysma vorming in muizen.

In **hoofdstuk 8** werd de rol van cathepsine K in structuurveranderingen van de vaatwand (vasculaire remodelering) verder bestudeerd. Hiervoor werd gebruik gemaakt van een model waarin de bloedstroom van één van de halsslagaders van apoE deficiënte of controle muizen werd afgesloten. Hierdoor zal de vaatwand zich aanpassen; door ophoping van cellen in de vaatwand ontstaat een zogenaamde neointima en het vat zal structuurveranderingen ondergaan (vasculaire remodelering). In de apoE deficiënte muizen bestaat de neointima met name uit een bepaald type ontstekingscellen, de macrofagen, terwijl in de controle/wildtype muizen de neointima met name bestaat uit gladde spiercellen. Uit de resultaten bleek dat in afwezigheid van cathepsine K de vorming van de macrofaag-rijke neointima in de apoE deficiënte muizen volledig geremd is, terwijl dit totaal geen effect had op de vorming van de neointima in de controle/wildtype muizen. Bovendien was het aantal circulerende (CD3-positieve) T-cellen verminderd en het aantal regulatoire T-cellen verhoogd in cathepsine K deficiënte apoE^{-/-} muizen, maar niet in de controle/wildtype muizen. Verdere analyse

in celkweken van geïsoleerde macrofagen toonde aan dat cathepsine K deficiënte macrofagen in het algemeen een hogere ontstekingsremmende activiteit bezaten dan controle macrofagen (M2a fenotype). Uit deze bevindingen kan geconcludeerd worden dat de verhoogde vasculaire remodeleringscapaciteit, zoals waargenomen bij apoE deficiënte muizen, bijna volledig geremd is in afwezigheid van cathepsine K. Dit wordt waarschijnlijk veroorzaakt door een correctie van de verhoogde ontstekingsactiviteit in apoE deficiënte muizen.

In **hoofdstuk 9** worden de bevindingen uit dit proefschrift bediscussieerd. Hoewel ontstekingsprocessen en vasculaire remodelering gemeenschappelijke kenmerken zijn van verscheidene vasculaire aandoeningen zoals atherosclerose en aneurysma vorming, stelden we vast dat een bepaalde ontstekingsremmende therapie alleen bescherming biedt tegen atherosclerose, maar niet effectief is tegen de ontwikkeling van een aneurysma. Ook bleek dat afwezigheid van de belangrijke protease cathepsine K beschermde tegen de vorming van een macrofaag-rijke neointima, maar niet tegen aneurysma vorming en het ontstaan van een gladde spiercelrijke neointima. Deze resultaten suggereren dat, hoewel ontstekingsprocessen en ECM afbraak onderdeel zijn van alle genoemde vaataandoeningen, een ontstekingsremmende therapie of een therapie gericht op voorkoming van ECM afbraak, niet even effectief was. Ondanks aanzienlijke bijwerkingen worden immunosuppressiva nog vaak toegepast/veel gebruikt in de klinische praktijk om orgaanafstoting tegen te gaan. Deze bijwerkingen staan een ruimer gebruik van deze geneesmiddelen in de weg. Er zijn echter wel experimentele ontwikkelingen die een meer specifieke remming van de betrokken moleculen mogelijk maakt. Dit opent perspectieven voor een aanpak, waarbij specifieke moleculen in bepaalde vaataandoeningen geremd kunnen worden en er minder risico is op bijwerkingen. Concluderend kan gesteld worden dat een ontstekingsremmende therapie of een therapie gericht op voorkoming van de ECM afbraak alleen effectief kan zijn als de therapie gericht ingrijpt op mechanismen die cruciaal zijn voor het ontstaan van de vasculaire aandoening.

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October 7th, 2009, Eindhoven

Curriculum Vitae

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3. Oral presentation in American Heart Association (AHA) Scientific Session 2008. Title: Cathepsin K deficiency does not affect murine aneurysm formation but reduces macrophage-rich neointima formation on ApoE^{-/-} background
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5. Oral presentation in Nederlandse Nefrologiedagen, 2004. Title: Substrain-selective glomerular expression of neuronal activity-regulated pentraxin(NARP) discriminates between remodeling and progression towards glomerulosclerosis after induction of anti-Thy-1nephritis(ATN) in rats

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1. Best presentation in Nederlandse Nefrologiedagen in Session Free communications 2004, Veldhoven, the Netherlands.
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3. Leiden Alumni Fund scholarship (LUF) in 2003
4. Partial tuition waiver awarded by Leiden University in 2003
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6. The Second prize in the 2000 National English Contest for college students, China.
7. The First prize in the 1997 English Speaking Competition in the Liaoning University of TCM, China.
8. Level eight certificate for non-professional piano player (grading system of China, level 10 is the highest)

List of Publications

Sylvia Heeneman, Marjo MPC Donners, **Lili Bai**, Mat JAP Daemen. Drug-induced immunomodulation to affect the development and progression of atherosclerosis: a new opportunity? Expert Review of Cardiovascular Therapy 2007. 5(2):345-364

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Lili Bai, Mathijs Groeneweg, Veronica Herias, Linda Beckers, Marjo Donners, Erwin Wijnands, Mat Rousch, Mat J.A.P. Daemen, Erik A.L. Biessen, Sylvia Heeneman . Differential effect of FK506 dosage on the development of atherosclerosis due to a dose-dependent shift in Th1/Th2 balance (in revision).

Lili Bai, Erwin Wijnands, Linda Beckers, Dirk Lievens, Mathijs Groeneweg, Mat Rousch, Tom Sejkens, Leon de Windt, Esther Lutgens, Mat J.A.P. Daemen, Ziad Mallat, Erik A.L. Biessen, Sylvia Heeneman. Leukocyte NFATC2 deficiency deteriorates rather than protects against atherosclerosis by facilitating memory T cell recruitment (in preparation).

Lili Bai, Guoping Shi, Suzanne P.M. Lutgens, Erwin Wijnands, Linda Beckers, Paul Saftig, Mat J.A.P. Daemen, Kitty Cleutjens, Erik A.L. Biessen, Sylvia Heeneman. Cathepsin K deficiency reduced carotid artery remodeling due to formation of a macrophage-rich lesion in apoE^{-/-} mice, but did not affect macrophage-poor lesion formation in wild type mice(in preparation).

Lili Bai. The Immunological effects of Traditional Chinese Medicine, Bu-Zhong-Yi-Qi decoction. The Journal of Jinzhou Medical College 2001. 22(4): 47-48. (In Chinese)

Lili Bai. Some Opinions on Creative Thought and Scientific Development. The Journal of Jinzhou Medical College 2001. 22(5):95-97. (In Chinese)

Figure 3.2 E Effects of low and high dose FK506 treatment on collar-induced atherosclerotic plaque development in common carotid arteries of apoE^{-/-} mice. Representative HE stained cross-sections of right carotid artery of control, low dose versus high dose FK506-treated mice, respectively.

Figure 5.2 Effects of leukocyte NFATC2 deficiency on atherosclerotic lesion formation in aortic arch. Representative HE stained cross-sections of aortic arch of control versus NFATC2^{-/-} transplanted mice, respectively.

Figure 5.3B Leukocyte NFATC2 deficiency significantly increased the amount of CD3-positive cells relative to total cells in advanced lesions compared with controls. Representative pictures of CD3⁺ staining of aortic arch of control and NFATC2^{-/-} transplanted mice are shown.

Figure 5.8F NFATC2 deficiency led to enhanced migration of CFSE+ cells to atherosclerotic aortic arch lesions. CFSE+ positive cells in sections from frozen aortic were quantified. Nuclei were counter-stained using Mounting Medium with DAPI. Overlay images were acquired using fluorescence microscope. Representative overlays of fluorescence image of the aortic arch from apoE^{-/-} mice that receive WT and NFATC2^{-/-} CFSE+ splenocytes respectively are shown.

Figure 7.2F representative photographs showing Elastica-von Giesson staining of aneurysms in apoE^{-/-} and catK^{-/-}//apoE^{-/-} mice. Both groups displayed complete medial breaks and subsequent lumen expansion (left panel) and adventitial thickening (right panel).

Figure 7.3H Effect of catK deficiency on collagen content in AAA lesions. Representative pictures showing Sirius red staining of AAA lesions from catK^{-/-}//apoE^{-/-} and apoE^{-/-} animals.

Figure 8.1A CatK expression in macrophage-rich (apoE^{-/-}) and macrophage-poor (WT) flow cessation-induced intimal lesions. Representative pictures of catK staining in wt and apoE^{-/-} mice are shown.

Figure 8.3A Effect of catK deficiency on intimal hyperplasia. Representative micrographs showing HE staining of intima lesions in WT, catK^{-/-}, apoE^{-/-} and catK^{-/-}/apoE^{-/-} mice.